Universidade de Aveiro Departamento de Química
2015

Ana Margarida Ferreira Martins

Extração de Ficobiliproteínas da macroalga vermelha *Gracilaria vermiculophylla*

Phycobiliproteins extraction from the red macroalga *Gracilaria vermiculophylla*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, realizada sob a orientação científica da Professora Doutora Sónia Patrícia Marques Ventura, Professora Auxiliar Convidada do Departamento de Química da Universidade de Aveiro, e co-orientação de Flávia Aparecida Vieira, Estagiária de Pós-Doutoramento do Departamento de Química da Universidade de Aveiro.

"The secret of making something work in your lives is, first of all, the deep desire to make it work;
then the faith and belief that it can work;
then to hold that clear definite vision in your consciousness and see it working out step by step,
without one doubt or disbelief."

EiLeen Caddy

o júri

presidente

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Palavras-chave Macroalgas, *Gracilaria vermiculophylla*, Extração Sólido-líquido, Ficobiliproteínas, R-ficoeritrina, Líquidos Iónicos

Resumo

Recentemente, o interesse pelos produtos obtidos de fontes naturais tem crescido em relação aos produtos sintéticos. Assim sendo, produtos extraídos de fontes naturais têm sido alvo de especial atenção pela indústria e pelo meio académico. Alguns desses compostos interessantes podem ser encontrados nas macroalgas vermelhas, nomeadamente a R-ficoeritrina, uma das ficobiliproteínas mais valiosas presentes na macroalga. Contudo, a maior dificuldade encontrada na extração e purificação desta proteína fotossintética está associada à necessidade de metodologias de extração e purificação mais eficazes, de mais baixo impacto económico e ambiental, capaz de remover as proteínas da biomassa, mantendo a sua estrutura conformacional e principais atividades.

Neste trabalho, um conjunto de parâmetros experimentais de extração foram otimizados, nomeadamente o solvente em causa. Várias soluções aquosas de líquidos iónicos foram testadas na extração de ficobiliproteínas a partir da macroalga vermelha *Gracilaria vermiculophylla*. Depois de otimizado o tempo de extração, a razão sólido-líquido, o solvente, pH e concentração de solvente, foi possível chegar a uma metodologia capaz de extrair mais 30% de ficobiliproteínas quando comparado com o método convencional reportado na literatura.

Keywords Macroalgae, *Gracilaria vermiculophylla*, Solid-Liquid Extraction, Phycobiliproteins, R-phycoerythrin, Ionic Liquids

Abstract In the past few years, there has been an increased demand for natural compounds over the synthetic ones. Thus, products extracted from natural sources have gained significant interest among industries and academia. Several of these interesting compounds are present in red macroalgae namely R-phycoerythrin, a phycobiliprotein. However, the major drawback is associated with the demand for more effective, with low economic and environmental impact extraction and purification methodology capable to remove the protein from the biomass, while maintaining its structure conformation and main activities. Therefore, the search for efficient extraction technologies is of utmost importance.

In this work, a set of different parameters of extraction was optimized, such as the solvent used. Aqueous solutions of ionic liquids were screened for the phycobiliproteins extraction from the *Gracilaria vermiculophylla*. Once optimized the time of extraction, the solid-liquid ratio, the solvent, the solvent concentration, and the pH, it was possible to design an efficient methodology capable to enhance the phycobiliproteins extraction in 30% when compared with the conventional extraction methodology.

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ABBREVIATIONS

A_{XYZ}	Absorbance at XYZ nm
ABS	Aqueous Biphasic System
ANOVA	Analyses of variance
DW	Dry Weight
IL	Ionic Liquid
kDa	Kilo Daltons
K_{ow}	Octanol-Water Partition Coefficient
Log K_{ow}	Logarithm (base 10) of the octanol-water partition coefficient
LSC	Luminescent solar concentrators
PCB	Peptide-linked Phycocyanobilin
PE	Phycoerythrin
PEB	Peptide-linked Phycoerytrobilin
PUB	Peptide-linked Phycourobilin
PXB	Peptide-linked Phycobiliviolin
SDS-PAGE	Sodium Dodecyl Dulfate Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
SLE	Solid–liquid-type extraction
SLR	Solid Liquid Ratio
SRM	Surface Response Methodology
$[C_2im][CH_3CO_2]$	1-ethylimidazolium acetate
$[C_2mim][CH_3CO_2]$	1-ethyl-3-methylimidazolium acetate
$[C_2mim]Cl$	1-ethyl-3-methylimidazolium chloride
$[C_4mim][CF_3CO_2]$	1-butyl-3-methylimidazolium trifluoroacetate
$[C_4mim][CF_3SO_3]$	1-butyl-3-methylimidazolium trifluoromethanesulfonate
$[C_4mim][CH_3CO_2]$	1-butyl-3-methylimidazolium acetate
$[C_4mim][CH_3SO_3]$	1-butyl-3-methylimidazolium methanosulfonate
$[C_4mim]Cl$	1-butyl-3-methylimidazolium chloride
$[C_4mim][DMP]$	1-butyl-3-methylimidazolium dimethylphosphate
$[C_4mim][N(CN)_2]$	1-butyl-3-methylimidazolium dicyanamide
$[C_4mim][SCN]$	1-butyl-3-methylimidazolium thiocyanate
$[C_4mim][Tos]$	1-butyl-3-methylimidazolium tosylate
$[C_4mpip]Cl$	1-butyl-1-methylpiperidinium chloride
$[C_4mpyr]Cl$	1-butyl-3-methylpyridinium chloride
$[C_4mpyrr][CH_3CO_2]$	1-butyl-1-methylpyrrolidinium acetate
$[C_4mpyrr]Cl$	1-butyl-1-methylpyrrolidinium chloride

$[C_6mim]Cl$	1-hexyl-3-methylimidazolium chloride
$[C_8mim]Cl$	1-methyl-3-octylimidazolium chloride
$[C_{10}mim]Cl$	1-decyl-3-methylimidazolium chloride
$[C_{12}mim]Cl$	1-dodecyl-3-methylimidazolium chloride
$[C_{14}mim]Cl$	1-methyl-3-tetradecylimidazolium chloride
[Ch]Ac	Cholinium acetate
[Ch]Cl	Cholinium chloride
$[N_{4,4,4,4}]Cl$	Tetrabutylammonium chloride
$[P_{4,4,4,4}]Cl$	Tetrabutylphosphonium chloride
β	Hydrogen bond basicity
λmax	Maximum absorption wavelength
‰	Per mille

1. INTRODUCTION

1.1 Marine resources: the particular case of macroalgae

The Earth's surface is mostly covered by water with oceans and seas representing over 70% of it. Living aquatic resources have called researchers' attention not only because of the large area they represent, but also due to their renewable and sustainable exploitation nature. Nowadays, aquatic resources already provide a significant number of products and services such as food, energy and bio-based products. Even so, it is known that the use of these resources can be improved by their deep and more fundamental understanding. Many national and international programs, namely CRER 2020 (regional program active for the Centre Region of Portugal, thus including Aveiro) and Horizon 2020 program, have been developed in order to optimize and maximize the sustainable exploitation of oceans, seas, and rivers. At the same time, it is intended to promote innovation taking into account the "blue biotechnology", science and economic development, through creation of industry and jobs [1].

In this context, algae have gained special importance due to their biotechnological potential. These marine matrices are a rich source of many highly valuable products/chemicals apart from the many applications they already have. Algae are photosynthetic organisms that can be found all over the world, predominantly oceans, rivers, lakes, streams, ponds, and marshes. Like plants, algae first need sunlight, carbon dioxide, and water so they can convert the energy of sunlight into chemical energy during photosynthesis [2,3]. The term "algae" is not easily defined. At the same time, it is consensual that they can range from small organisms, made up of just one cell, known as microalgae, to multicellular organisms with many differentiated forms, known as macroalgae or seaweeds [2,3].

1.1.1 Macroalgae

Macroalgae are multicellular fast-growing organisms which can reach sizes up to 60 meters in length and can be found in salt or fresh water [3]. The use of macroalgae was spread all over the world and under different forms and applications, namely as animal food, remedies, and fertilizers [4]. From the technological point of view, macroalgae have been used as sources of natural colorants, phycocolloids, thickening and gelling agents, those typically applied in food industries [5,6]. More recently, the interest by this marine biomass has been increased since the biotechnological potential of different

species is known. Many macroalgae grow in extreme conditions, thus producing a wide variety of primary and secondary unique metabolites [7]. Its exploitation is now seen as a potential source of new biochemicals and bioactive drugs, when considered their many biological activities. Macroalgae produce carotenoids, terpenoids, chlorophylls, phycobilins, polyunsaturated fatty acids, polysaccharides, vitamins, sterols, tocopherol and phycocyanins, among others [7]. These phytochemicals have well-known health benefits, namely their anticancer, anti-inflammatory, antioxidant, antitumoral, and antimicrobial activities, anticoagulant properties, possible anti-obesity effect and reduced risk of diabetes type II [6].

Macroalgae have been identified and grouped based on their pigmentation as green (*Chlorophyceae*), red (*Rhodophyceae*) and brown seaweed (*Phaeophyceae*) [3]. The green color of green seaweeds results from the content of chlorophylls *a* and *b* found in the macroalgae composition. The brown coloration of brown seaweed is related with the dominance of pigments such as fucoxanthin masking the presence of other pigments such as chlorophylls *a* and *c*, β-carotenes, and other xanthophylls. A similar behavior describes the red seaweed, since the presence of red compounds like phycoerythrin (PE) and phycocyanin hide the color provided by chlorophyll *a*, β-carotene, and a number of unique xanthophylls [8].

1.1.2 Red Macroalgae

There are about 8000 species of red macroalgae, most of which grow in aquatic environments. These are found in the intertidal and in sub-intertidal to depths of up to 40, or occasionally 250 meters [8]. Some authors refer this macroalga class as the most important source of many biologically active metabolites, with a wide range of biological activities, namely cytotoxic, antiviral, anti-inflammatory, antimalarial, free radical scavenger, neurophysiological, insecticidal, and antimicrobial activities, among others [8].

The biochemical composition of red macroalgae varies with species and potentially with location, seasonality and growth conditions [7,9]. Proteins are one of the most interesting groups of compounds found mainly in red macroalgae due to their applications in the health sector [7]. Some red macroalgae are known for their high proteinic content, in some cases containing higher protein amounts than the

conventional proteinic-rich foods namely soybean, cereals, eggs, and fish [10]. As an example, *Porphyra tenera* was reported in 1984 for its high protein content of 47% in DW. However, the class and abundance of proteins, as well as the amino-acid content, are parameters that strongly depend on the species and season [11], as described for *Palmaria palmata* (protein content ranging from 8% to 35% [9]), for *Gracilaria gracilis* (protein content ranging from 45% in January to 31% in July of DW [12]) and for *Grateloupia turuturu* (protein content ranging from 14% to 27.5% of DW, in the summer and winter [13], respectively).

1.2 Phycobiliproteins

Phycobiliproteins are the major photosynthetic pigments found in red macroalgae, cyanobacteria, and cryptomonads, a unicellular eukaryotic algae [14]. These pigments are predominant light-harvesting pigment-protein complexes organized *in vivo* in supramolecular structures called phycobilisomes [9]. Phycobilisomes are located in the stroma, on the external structure of thylakoid membranes (**Figure 1**).

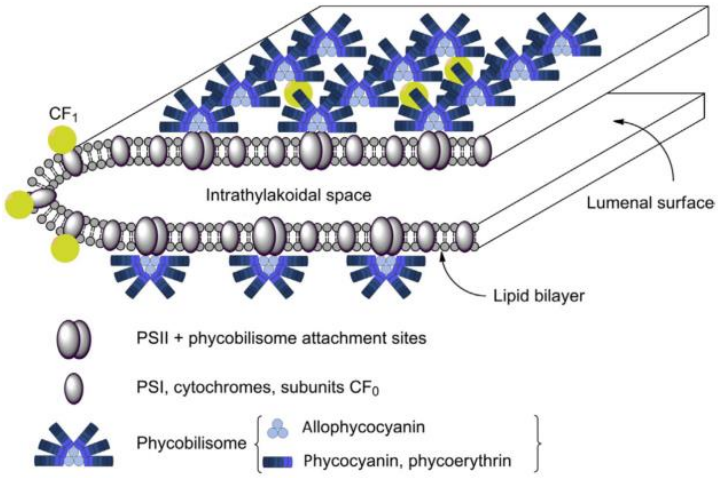

Figure 1. Schematic representation of the phycobilisomes in the thylakoid membrane. CF: coupling factor; PS: photosystem. Adapted from [9].

The association of phycobiliproteins allows simultaneously the transfer of light energy (**Figure 2**) and the survival of living organisms at low light intensities [9]. In fact, the phycobilisomes work as energetic funnels, in which morphology and energetics are matched to allow the energy transfer from any one of the hundreds of chromophores to

the reaction centers [15]. They are able to absorb light in spectral zones where chlorophyll *a* cannot, thus transmitting this energy to the photosystem II reactive center with an efficiency greater than 90% [9,14].

Figure 2. Light energy transfer pathway in phycobilisomes [9].

All phycobiliproteins have the same basic unit: a heterodimer of α and β subunits, conventionally referred as *monomer*. α and β subunits are chains of approximately 160-180 amino-acid residues. Three of these monomers are associated to form the basic building block of phycobilisomes, the $(\alpha\beta)_3$ trimer. The trimers assemble into hexamers with the help of linkage polypeptides, as depicted in **Figure 3** [9,16].

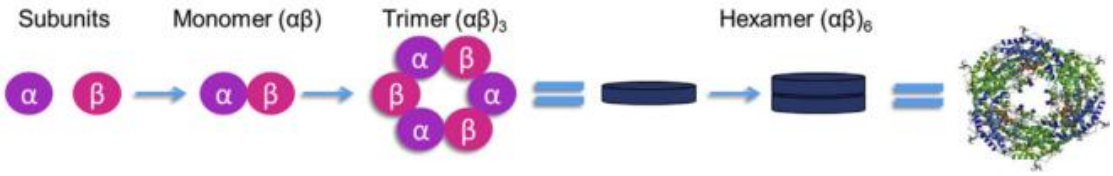

Figure 3. Schematic representation of a phycobiliprotein subunit assemblage. Adapted from [9].

The hexamers synthesis is the starting point to the formation of the whole structure. It is usual to divide the morphology of phycobiliproteins into two different structures; the core and the rods (**Figure 4**). The core is composed by a set of three allophycocyanin molecules, each composed of four linked hexamers. They are placed in the middle of the phycobilisomes, being connected to the thylakoid by polypeptides. The rods have a shape of a stick and are made up of hexamers of phycoerythrin, phycoerythrocyanin or phycocyanin and also linked by polypeptides [9].

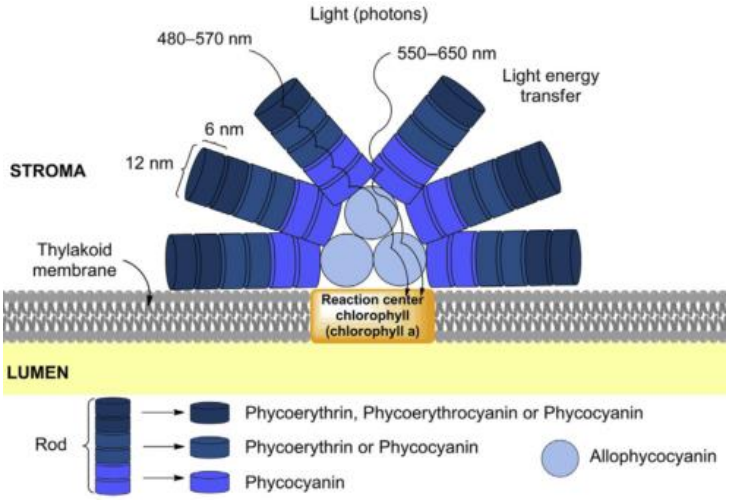

Figure 4. Schematic representation of a the common phycobilisome structure in red macroalgae [9].

In the phycobilisomes, linkage polypeptides represent around 15% of the total protein content and have an important role, the fixation to the thylakoid membrane, stabilization of the structure, and ensure the cohesion between phycobiliproteins. In fact, phycobiliproteins and linkage polypeptide have different features: phycobiliproteins are acidic and hydrophilic structures, while the linkage polypeptides are alkaline and of hydrophobic nature [17].

Stepping back to the monomers, each one can carry either one, two or three chromophores depending on the molecular species. Chromophore is the part of a molecule responsible for its color, leading to differences in their optical properties. In the case of phycobiliproteins, the chromophores are called phycobilins (**Figure 5**). These specific structures are made up of four different kinds of open-chain tetrapyrroles that are covalently bound to cysteins of the apoproteins *via* thioether bonds [15,16]. The phycobilins are the purple-colored phycobiliviolin (PXB), the blue-colored phycocyanobilin (PCB), the red-colored phycoerythrobilin (PEB) and turquoise-blue-colored represented by the phycourobilin (PUB).

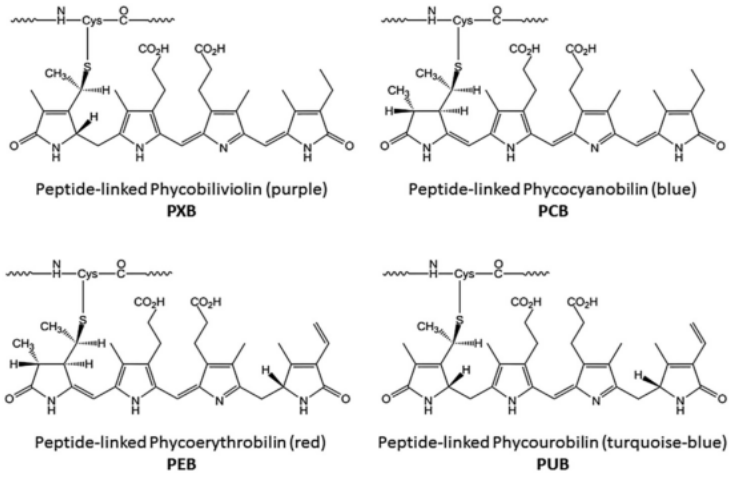

Figure 5. Molecular structure of the four chromophores of phycobiliproteins [9].

The phycobiliproteins are usually classified into four classes based on their light absorption behavior: phycoerythrin with maximum absorption wavelengths (λmax) ranging between 540 and 570 nm; phycocyanins (λmax = 610-620nm), allophycocyanin (λmax = 650-655 nm) and phycoerythrocyanin (λmax = 560-600 nm) [18]. All these molecules differ in the amino-acid sequence, number of chromophores *per* subunit and type of chromophores (**Table 1** summarizes the composition of the individual phycobiliproteins).

Table 1. Molecular species of phycobiliproteins. Adapted from [16].

Phycobiliproteins	**Subunit composition**	**Chromophores in** α-subunit	β-subunit	γ-subunit
Allophycocyanin (APC)				
APC	$(\alpha\beta)_3$	1 PCB	1 PCB	
APC-B	$(\alpha\beta)_2\alpha^{APB}\beta$	1 PCB	1 PCB	
Phycocyanin (PC)				
C-PC	$(\alpha\beta)_3$	1 PCB	2 PCB	
R-PC	$(\alpha\beta)_3$	1 PCB	1 PCB, 1 PEB	
Phycoerythrocyanin	$(\alpha\beta)_3$	1 PXB	2 PCB	
Phycoerythrin (PE)				
C-PE	$(\alpha\beta)_3$	2 PEB	3 PEB	
b-PE	$(\alpha\beta)_6\gamma$	2 PEB	3 PEB	2 PEB, 2 PUB
B-PE	$(\alpha\beta)_6\gamma$	2 PEB	3 PEB	2 PEB, 2 PUB
R-PE	$(\alpha\beta)_6\gamma$	2 PEB	2 PEB, 1 PUB	2 PEB, 2 PUB

Allophycocyanin is mostly defined by a trimer $(\alpha\beta)_3$ with a molecular weight of approximately 110 kDa. Both α and β subunits have PCB chromophores which confer the blue color to this phycobiliprotein. Its maximum absorption wavelength is described at 650 nm, corresponding approximately to the second maximum of chlorophyll *b*. Allophycocyanin is one of the core constituents of macroalgae and it possesses an intermediary function in the energy transfer mechanism through the chlorophyll *a* reaction centers [9]. The content in allophycocyanin is the less significant in the phycobiliproteins, representing only 5% of the soluble proteins in *Porphyridium cruentum*. Phycocyanin can be found in some organisms like cyanobacteria, Rhodophyta and Cryptophyta. Two types have been described: C-phycocyanin, which characterizes the cyanobacterial pigments; and R-phycocyanin, thus representing the red-macroalgal biliproteins [9,15]. This chromophore is also identified by its blue color and both α and β subunits possessing PCB chromophores but, in this case, the β subunit has two chromophores (**Table 1**).

Phycoerythrocyanin was firstly described in 1976 by Bryant and co-workers [19]. This phycobiliprotein is known by its purple color. Phycoerythrocyanin can be found in both $(\alpha\beta)_3$ or $(\alpha\beta)_6$ structural forms. The PXB is a chromophore specific of phycoerythrocyanin and it is located on α subunit while the β subunit carries two PCB chromophores [9]. The protein and chromophore structures of phycoerythrocyanin and phycocyanin are very similar, except in the α-subunit with the substitution of phycobiliviolin for phycocyanobilin [16].

Phycoerythrin is the main pigment found in red macroalgae but it can also be found in cyanobacteria. Depending on the specie, different forms of PE can occur: R-phycoerythrin (R-PE), for Rhodophyta; B-phycoerythrin (B-PE) and b-phycoerythrin (b-PE), for Bangiales; C-phycoerythrin (C-PE), for cyanobacteria. B-PE and R-PE are the most abundant phycoerythrins in red macroalgae [9,15]. PE is composed of $(\alpha\beta)_6\gamma$ complexes with a molecular weight ranging from 240 to 260 kDa, except C-PE which do not have any γ-subunit [9,16]. **Figure 6** represents the UV-Vis spectra of phycobiliproteins. Phycobiliproteins exhibit different behaviors in what concerns their light absorption characteristics, being thus a good differentiate method. Exceptionally, R-phycoerythrin has a special absorption spectrum in its native state with a three-peak absorption maxima at 565, 539 and 498 nm [20].

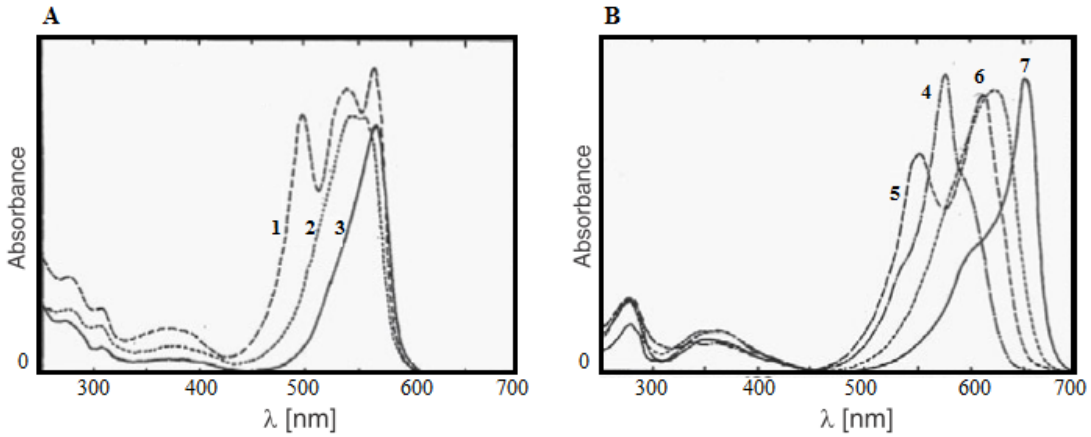

Figure 6. Absorption spectra of phycobiliproteins: **(A)** Phycoerythrin (1 = R-PE, 2 = B-PE, 3 = C-PE); **(B)** Other phycobiliproteins (4 = Phycoerythrocyanin, 5 = R-phycocyanin, 6 = C-phycocyanin and 7 = Allophycocyanin) [15].

1.3. R-Phycoerythrin (R-PE)

1.3.1 Structure and Stability

R-phycoerythrin (**Figure 7**) is a $(\alpha\beta)_6\gamma$ complex composed of subunits with different molecular weights: α, 18-20 kDa; β, 19.5-21 kDa; and γ, 30 kDa [20]. The increment of the γ subunit in R-phycoerythrin, in comparison with other phycobiliproteins, has an important role in this protein. Since the γ subunit is located in the center of the molecule, it links the $(\alpha\beta)_3$ trimers conferring a high stability to this protein [21].

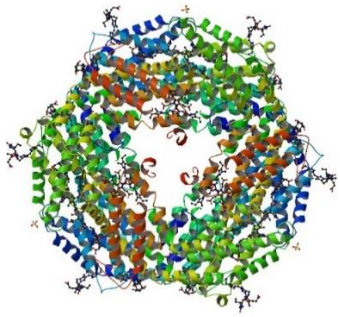

Figure 7. Crystal Structure of R-phycoerythrin of *Gracilaria chilensis* at 2.2 Angstroms (protein chains are colored from the N-terminal to the C-terminal using a spectral color gradient) [22].

Several authors already studied the stability of R-phycoerythrin according to different parameters. Munier et al. (2014) [18] reported the stability of R-phycoerythrin extracted from the red macroalgae *Grateloupia turuturu* in different conditions of temperature, pH, and light exposure time (**Figure 8**).

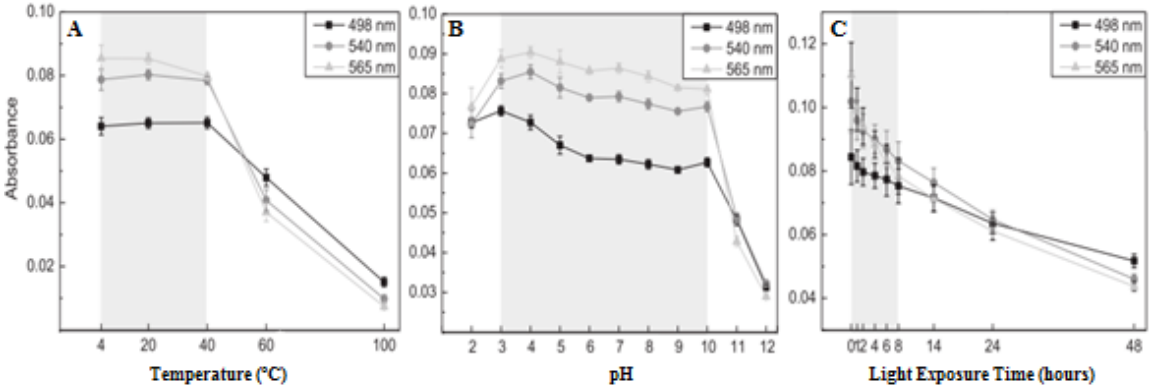

Figure 8. Effect of: **(A)** temperature; **(B)** pH; and **(C)** light exposure time on absorption spectra of aqueous extracts. The area in grey represents no statistical difference. Adapted from [18].

Concerning the temperature (**Figure 8**A), no significant modifications were reported up to 40ºC, regarding the absorbance or the fluorescence spectra, however it was possible to conclude a decrease in the intensity of the protein at 60ºC and no absorption and fluorescence spectra at 100ºC. This loss of protein stability may be associated to the decrease in the amount of the α-helix, disturbing the pigment stability [18]. Towards pH variations (**Figure 8**B), R-phycoerythrin seems do not be disturbed at pH values ranging from 4 to 10, which is proved by the unchangeable color [18]. At pH values between 2 and 3, the solutions of R-phycoerythrin were purplish, while at pH values of 11 and 12, the intensity of the color is decreased until the solutions became colorless. This can be explained by the fact that significant changes in pH may result in the disturbance of the electrostatic properties and hydrogen bonds involved in the protein association, that can induce changes in the chromophore structure [18]. Very low pH levels may even cause the dissociation of the trimers to monomers, monomers into individual subunits, and partial unfolding of the subunits [23]. At last, all the R-phycoerythrin absorption peaks seem to suffer the impact of the time light exposure (**Figure 8**C). After 48 h of light exposure, the solutions tested became colorless and there is a clear reduction *at circa* of 70% in the R-phycoerythrin concentration [18]. The authors concluded that the peak at 498 nm, corresponding to phycourobilin chromophore, is more stable than the 540 nm and 565 nm, corresponding to the phycoerythrobilin chromophore, which is in accordance with recent studies [18,20]. This phenomenon is associated with the chromophore structure and more likely with the double bonds in the rings of these structures, making PUB stronger than PEB. Briefly, Munier and co-workers [18] proved

the R-phycoerythrin stability for non-extreme environmental conditions, without denaturation and consequent decline of R-phycoerythrin concentration.

Galland-Irmouli and collaborators (2000) also studied the stability of R-phycoerythrin from the red macroalga *Palmaria palmate* [20]. R-phycoerythrin displayed a good stability for a range of pH values between 3.5 and 9.5 and for temperatures up to 60ºC, since there were no significant modifications in color and fluorescence spectra of the protein. This range of pH in which it was proved the good stability of R-phycoerythrin is in concordance with Kawsar and co-workers for R-phycoerythrin from *Amphiroa anceps* [24].

1.3.2 Techniques to enhance the extraction of R-phycoerythrin

Actually, the knowledge about the variations of the biochemical composition levels according to species, geographic area, season, and environmental conditions are thus relevant information to properly characterize and control the manipulation of red macroalgae. This information can lead to the development of strategies to enhance the production of compounds with significant value [25], namely R-phycoerythrin. For the specific case of R-phycoerythrin, there are some published studies in which it was determined the R-phycoerythrin content under different environmental conditions such as salinity, temperature, light exposure, and nitrogen uptake for different species of red macroalgae [25–29]. Baghel and collaborators (2014) [25] studied the influence of different degrees of salinity (25-40‰) and temperature (20-35ºC) in the daily growth rate and R-phycoerythrin and R-phycocyanin content of the marine red macroalga *Gracilaria crassa*. The main results showed that different optimal conditions should be performed in order to enhance the production either in R-phycoerythrin or R-phycocyanin. The maximum daily growth was reached at 25ºC and 35‰ of salinity, being these conditions representing the highest R-phycoerythrin content (444.7±1.9 $\mu g.g^{-1}$ fresh weight basis) achieved, while the best conditions for R-phycocyanin were represented by 30‰ of salinity and 30ºC [25]. Apart from both temperature and salinity, one of the most important parameters to be controlled is the nitrogen source and concentration. An environment deficiency in micronutrients may have a dramatic effect on the cellular pigmentation, and consequently on the quantity of R-phycoerythrin present in the macroalgae biomass. When the nutrient is nitrogen, its deficiency leads to

a biliprotein synthesis inhibition [20]. Macroalgae can use a wide variety of nitrogenous sources to fulfill their requirements in nitrogen, namely ammonia, nitrate, urea, amino-acids, and nucleosides, being NO_3^- and NH_4^+ the primary sources [27]. In general, the nitrogen addition is responsible for the increase of the photosynthetic activity and thus, for the growth of macroalgae, since it is an essential element incorporated in to many organic macromolecules (proteins, nucleic acids and pigments). Kim and co-workers [27] proved that the specific growth rate and R-phycoerythrin content increased with the increase of the ammonium concentration for all red macroalgae studied. However, for some species, namely *Porphyra leucostict*, *Porphyra linearis*, and *Porphyra umbilicalis*, the R-phycoerythrin concentration was affected by temperature at high ammonium concentration. Other studies report the influence of light sources of different wavelengths in the red macroalgae metabolism, growth and pigmentation composition. Godínez-Ortega et al. (2008) found that cultures of *Halymenia floresii* exposed to blue and red light were stimulated to synthetize phycoeryhtrin [26].

1.3.3 Applications

Nowadays, there is an increasing demand and preference for natural products and compounds. Due to their characteristics, R-phycoerythrin and other phycobiliproteins have gained special significance in many different commercial sectors. The high solubility in water, stability, and proteinic nature has lead much attention in the food, pharmaceutical, cosmetic, and textile industries [9,18]. In the food industry, these structures are mainly applied as red fluorescent colorants especially for jellified desserts and dairy products and for the production of functional food, a recent, but very promising field. R-phycoerythrin is also pointed out in some areas related with medicine, namely clinical medicine, diagnosis, and biomedical research [30], due to their excellent optical and spectroscopic properties, high absorption coefficient, and high fluorescence yield. It is especially used as fluorescent probe in flow cytometry, microscopy, immunochemistry, and in different biomedical reagent formulations [18]. Moreover, R-phycoerythrin can be used as an internal marker in electrophoretic techniques and size gel exclusion chromatography since R-phycoerythrin chromophore groups have a deep rose color and R-phycoerythrin subunits have low molecular weight [14]. It also possesses some biological activities, namely their role as antioxidant,

antidiabetic, immunosuppressive and antihypertensive chemicals. It was also reported their anti-cancer activity, in which R-phycoerythrin can help to improve the selectivity of photodynamic therapy, activity tested recently in mouse tumor cells and human liver carcinoma cells [9,31].

Beside all referred applications, a novel and promising application has emerged for R-phycoerythrin and other phycobiliproteins. They can be used in the energy field, regarding the production of organic solar cells of photovoltaics panels, called "dye-sensitized solar cells". These have the ability to convert photons into a flow of electrons. It was already developed a similar concept using chlorophylls (**Figure 9**) and anthocyanins [32] for the same propose consisting of improve the absorption and conversion of sunlight into energy and at the same time making this process more environmentally friendly, safer and cheaper [33–35]. Despite the application of chlorophylls [36,37] in dye-sensitized solar cells, the luminescent solar concentrators (LSC) are also devices with crescent importance in the energy field, which can be improving the advantageous nature of the photovoltaic panels, since these display higher efficiencies at lower costs [38]. Nevertheless, the use of phycobiliproteins can mean an improvement comparing with the devices made of chlorophyll due to their very high photoluminescent efficiency, high absorption coefficients and stability [39,40]. Mulder et al. demonstrate the efficiency of the LSC employing phycobilisomes [41].

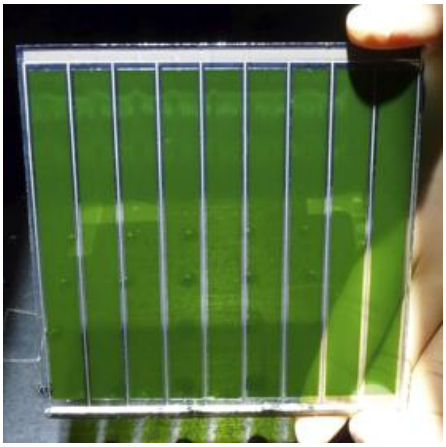

Figure 9. Dye-sensitized solar cells made up with chlorophylls [35].

1.4 R-phycoerythrin Extraction Procedures

The initial condition of the macroalgae can range from fresh, frozen or freeze-dried [42,43]. In order to increase the contact surface between biomass and the solvent, the macroalgae are usually grounded in liquid nitrogen and the resulting powder is then homogenized in the chosen buffer. The main objective of grinding in liquid nitrogen is to help the disruption of the cell wall, which is the biggest obstacle to access and extract the proteins from the macroalgae solid biomass [14]. Attempting to the water soluble behavior of R-phycoerythrin, it can be extracted by soaking seaweed in water for one or more days. The extraction process occurs due to the osmotic shock in the cells. Nevertheless, this procedure exhibits some significant disadvantages, since this type of extraction is done in long-time terms and thus, the proteases can promote the partial degradation of R-phycoerythrin [9]. Several procedures used (sodium) phosphate buffer (5-50 mM, pH 7) as solvent to extract the R-phycoerythrin [18,20,44].

Since processes of grinding in liquid nitrogen are expensive for the industrial scale and with lower efficiency, enzymatic hydrolysis of the cell wall has been suggested as an alternative method to extract the proteins [9]. The presence of high amounts of polysaccharides is reported as a limiting step regarding the contact between the solvent and the protein content, thus reducing significantly the extraction efficiency. Polysaccharides, such as xylans, agar, carrageenan or alginates, are present in large quantities in the cell wall of many red macroalgae. Furthermore, the strong covalent bonding between mix-linked xylan and glycoprotein complexes, already reported for *P. palmata*, is also an obstacle to overcome [45,46]. However, enzyme treatments have been recently demonstrated to be an effective method for the R-phycoerythrin extraction [9,46]. Dumay et al. (2013) found R-phycoerythrin extraction yield is 62 times greater than without enzyme treatment [46]. Other conventional techniques are normally associated to the extraction process, namely centrifugation aiming at to remove insoluble particles, to obtain a water soluble extract where phycoerythrin is dissolved. **Table 2** shows a compilation of R-phycoerythrin extraction procedures already established and their respective extraction yields.

Table 2. Enumeration and comparison of extraction conditions and respective R-phycoerythrin yield of extraction from different red macroalgae species. The data are arranged by publication date.

Extract	Initial Condition	Extraction Conditions				R-PE Yield in Crude Extract ($mg.g^{-1}$)	Ref.
		Solvent	Solid-Liquid Ration (m/v)	Temperature (ºC)	Time		
Audouinella saviana	Fresh and Cryogrounded	Phosphate buffer 5 mM (pH 6.8)	1:5	4	24 h	nd	[47]
Corallina officinalis	Fresh and Cryogrounded	20 mM sodium phosphate buffer (pH 7.1)	150 g alga[a]	4	nd	nd	[48]
Ceramium isogonum	Air-Dried	Potassium Phosphate buffer (pH 6.8) + 1.5% sodium nitrate	15 g alga*[a]	Freeze-Thaw cycles of -20 and 4°C	nd	nd	[49]
Gracilaria longa	Fresh and Cryogrounded	Potassium Phosphate buffer 50 mM (pH 6.8)	nd	4	24 h	nd	[44]
Palmaria palmata	Freeze-dried and Cryogrounded	Phosphate Buffer 20 mM (pH 7.0)	1:4*	nd	3 x 1 min (at 24 000 rpm)	2.26*	[20]
Gracilaria verrucosa	Fresh	Fresh Water	1:1	Room Temperature	Overnight	1.50	[50]
			1:2			1.27	
			1:3			1.15	
Corallina elongata Ellis & Solander	Freeze-dried	Sodium Phosphate buffer 10 mM w/ 100 mM NaCl (pH 7)	1:3*	nd	nd	0.6*	[51]
Polysiphonia urceolata	Frozen	Sodium Phosphate buffer 20 mM (pH 7)	30 g alga[a]	20	2 h	33.3	[43]
Polysiphonia urceolata Grev	Frozen	Distilled Water	1:3	4	24 h	1.57	[52]
Porphyra haitanensis	Fresh leafy gametophyte	Distilled Water	5:100	Freeze–Thaw cycles of -25 and 4°C (3×)	nd	18	[53]
	Fresh filamentous sporophyte		5:200			12	
Grateloupia turuturu	Freeze-dried	Potassium Phosphate	nd	nd	nd	2.75*	[42]

			buffer 20 mM (pH 7.1)					
			Distilled Water				2.79*	
	Fresh		Potassium Phosphate buffer 20 mM (pH 7.1)				1.07*	
			Distilled Water				1.00*	
Grateloupia turuturu	Freeze-dried and Cryogrounded		Distilled Water	129:1000*	Low Temperature	nd	0.38*	[54]
Heterosiphonia japonica	Fresh		Phosphate buffer Saline 50 mM (pH 7.4)	1:4	4	10 min in a Ultrassonic Cell Disruptor (350 W)	nd	[55]
Porphyra yezoensis Ueda	Fresh and Grounded		Phosphate buffer 10 mM (pH 6.8)	7:75	Freeze–Thaw cycles of -25 and 4°C (×3)	nd	6.06	[56]
Amphiroa anceps	Fresh and Crushed		Phosphate buffer saline 50 mM w/ 150 mM NaCl (pH 7.4)	7:100*	4	nd	47.14*	[24]
Porphyra yezoensis	Frozen		Sodium Phosphate buffer 50 mM (pH 6.8)	1:5	nd	Overnight	nd	[57]
Palmaria palmata	Freeze-dried	Medium pieces	Acetate buffer 50 mM (pH 5) + Xylanase	1:100*	40	nd	3.1*[b]	[46]
	Fresh						3.3*[b]	
	Freeze-dried	Small pieces					1.15*	
	Fresh						4.36*[b]	
	Freeze-dried	Cryogrounded					3.8*[b]	
	Fresh						3.6*	
Gracilaria lemaneiformis	Fresh and Grounded		Phosphate buffer 10 mM (pH 6.8)	10:25	Freeze–Thaw cycles of -25 and 4°C	Overnight	1.73	[30]

Portieria hornemannii	Fresh	Phosphate buffer 20 mM (pH 7.2)	10:25	Freeze-Thaw (×3)		1.23	[58]
Grateloupia turuturu	Freeze-dried and Cryogrounded	Sodium Phosphate buffer 20 mM (pH 7.1)	1:20	4	20 min	nd	[18]
Porphyra yezoensis Ueda	Dried	Phosphate buffer saline 50 mM (pH 6.8)	1:5	nd	nd	2.0[b]	[59]

Note: *Refers to Dry Weight; [a] No reference about the volume of buffer used; [b] Found by graph values; **nd**: no data.

Solutions of purified phycobiliproteins are expensive and their prices are still increasing. Their global market is being estimated at US$50 million with estimated yearly growths of 10% [60]. Considering the European scenario, the lab market prices of the native pigment (≤ 30% of protein) are in the range of 140-550€/mg but can reach 1300€/mg for cross-linked pigments (buffered solutions with antibodies or other fluorescent molecules). The prices change with companies [60], quantity supplied, purity, protein stability and source. Different methodologies have already been proposed to isolate and purify R-phycoerythrin. Usually, these procedures of purification are a combination of some techniques to reach better purity ratios. The purity of R-phycoerythrin is (usually) measured by a ratio of absorbance values represented by A_{565}/A_{280}. It is normally considered as an indication of the purity of R-phycoerythrin towards all remaining contaminant proteins also removed from the solid seaweed in the extraction process, and how bigger this value is, the purest is the liquid extract in R-phycoerythrin. Taking into consideration all information here described, it is concluded that these obstacles make pure R-phycoerythrin extracts become more expensive and are demanding the search for the development of new and more simple, faster and at the same time, efficient extraction technologies. This, allied with the increase interest of the industrial and academic communities for the marine resources (important demands of the *Horizon 2020* and *CRER 2020*) has been attracted researchers to develop best R-phycoerythrin extraction processes.

1.5 Ionic Liquids (ILs)

Ionic liquids (ILs) are salts usually composed by large organic cations and organic or inorganic anions. Due to their low-charge density and low symmetric ions, ILs melting point are, by general definition, below 100ºC [61]. These salts are known as “designer solvents” due to their “tunable” nature - change of their properties through the different possible combinations of their ions. This is a very important and supplementary advantage since thermophysical properties, biodegradation, toxicological properties, hydrophobicity and solution behavior can be adjusted according to the IL application [61,62]. ILs can be designed for a particular process, allowing thus the manipulation of their extraction capabilities for specific biomolecules [63]. The choice of the ILs’ cation and anion (**Figure 10** and **Figure 11**) can determine their thermophysical properties,

namely their water miscibility [64–66]. Only in the past few years these compounds have been extensively studied due to the synthesis of water-stable ILs, as well as task-specific compounds [61]. Thus, the development and emergent applications of new ILs have significantly increased, which is clearly represented by the number of publications regarding ILs which have been exponentially increasing since 1990, with 2500 publications, until now, with almost 130 000 publications. ILs are associated to some particular characteristics due to their ionic character. They are defined by some physico-chemical advantages over conventional and molecular organic solvents. These advantages include high solvation ability, high chemical and thermal stability, high selectivity, excellent microwave-absorbing ability, broad liquid temperature range, and high ionic conductivity [61,62,67]. Besides, negligible flammability and vapor pressure which give them the label of "green solvent" [68]. Moreover, many organic, organometallic and inorganic compounds can be dissolved in ILs. All these features make them promising alternatives to substitute the volatile organic compounds generally applied in the biocatalysis [67], in organic synthesis [69], in polymerization [70], in the dissolution of biomaterials [71] and, also very important, in extraction processes [61].

Currently, around 300 ILs are commercially available, and 1000 ILs are described in literature [72]. From those, several can be potentially applied as extractive solvents for different (bio)molecules, namely those applied in the formulations of different liquid-liquid extraction technologies [63].

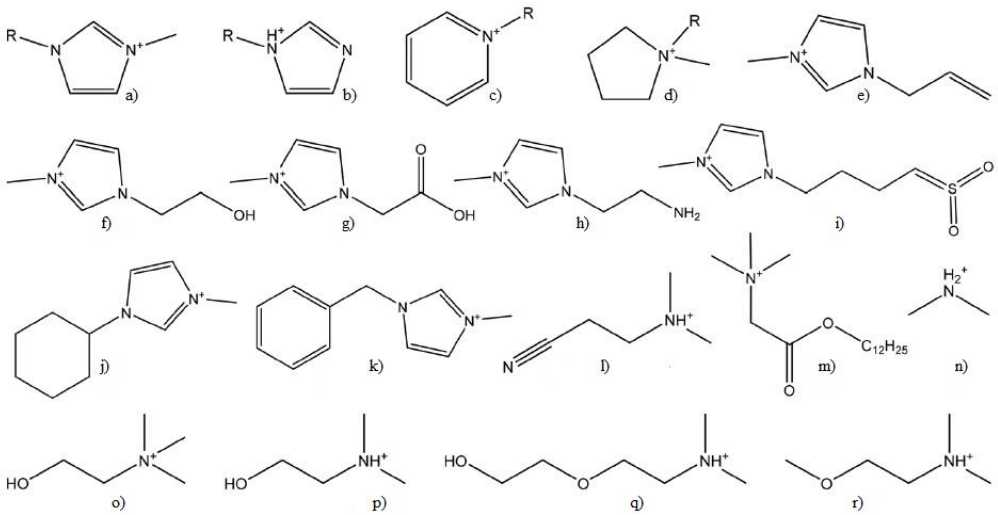

Figure 10. IL' cations: a) 1-Alkyl-3-methylimidazolium; b) 1-alkylimidazolium; c) 1-alkylpyridinium; d) 1-Alkyl-1-methylpyrrolidinium; e) 1-allyl-3-methylimidazolium; f) 1-hydroxyethyl-3-

methylimidazolium; g) 1-carboxymethyl-3-methylimidazolium; h) 1-propylamine-3-methylimidazolium; i) 1-(4-sulfonylbutyl)-3-methylimidazolium; j) 1-cyclohexyl-3-methylimidazolium; k) 1-benzyl-3-methylimidazolium; l) N,N-dimethyl(cyanoethyl)ammonium; m) 2-(dodecyloxy)-N,N,N-trimethyl-2-oxoethanaminium; n) N,N-dimethylammonium; o) cholinium; p) dimethylcarbamate; N,N-dimethylethanolammonium; q) N,N-dimethyl-N-(2-hydroxyethoxyethyl)ammonium; r) N,N-dimethyl(2-methoxyethyl)ammonium [61].

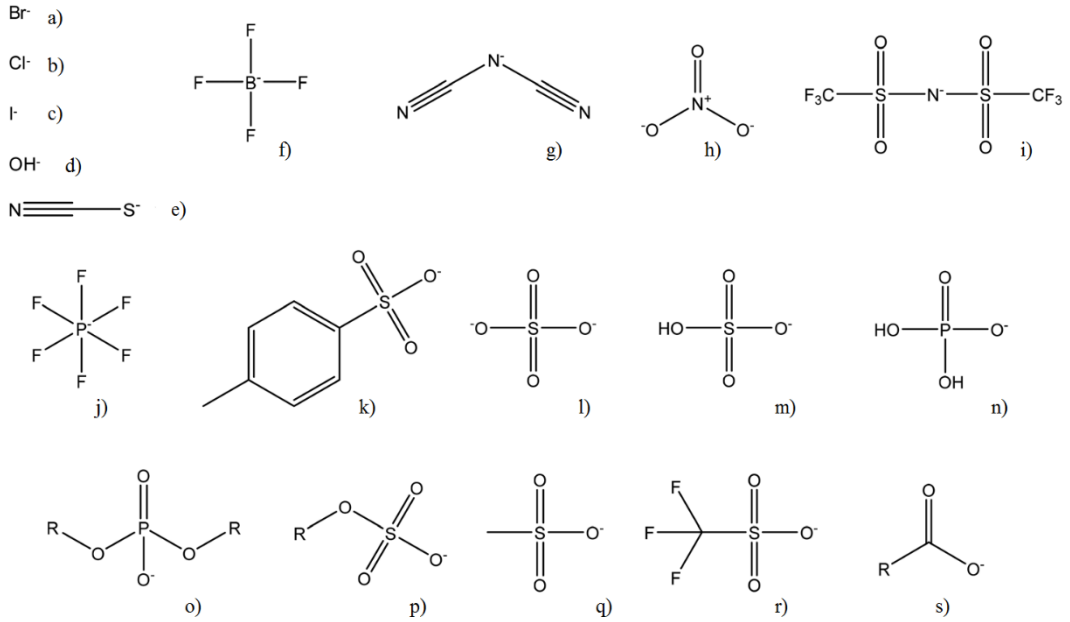

Figure 11. IL' anions: a) bromide; b) chloride; c) iodide; d) hydroxide; e) thiocyanate; f) tetrafluoroborate; g) dicyanamide; h) nitrate; i) bis(trifluoromethylsulfonyl)imide; j) hexafluorophosphate; k) tosylate; l) sulphate; m) hydrogenosulphate; n) dihydrogenophosphate; o) dialkylphosphate; p) alkylsulphate; q) methylsulfonate; r) trifluoromethanesulfonate; s) alkylcarboxylate [61].

All high-value compounds extracted from solid biomass with ILs start with a step of solid–liquid extractions (SLE) to remove the target chemicals form the solid residue. In order to reach higher extraction yields, some of these SLE processes were coupled to alternative techniques, in particular microwave-assisted extraction and ultrasound-assisted extraction [61]. Several compounds were already studied regarding their extraction from various biomass matrices and applying ILs, namely alkaloids, flavonoids, terpenoids, aromatic compounds, phenolic acids, lipids, and natural mixtures, such as essential oils, suberin and saponins [61]. This first step of extraction

contributes for the extraction of other chemicals usually designated by contaminants and, depending on the complexity of the biomass, these contaminants can be the principal chemicals present in the liquid crude extract formed, but from the point of view of concentration. It is in this context that the search for technologies with the capacity to separate the main contaminants from the valuable target compounds has become essential. ILs can play an important role in this scenario, namely considering their use as solvents applied in the formulations of aqueous biphasic systems (or commonly designed ABS).

1.6 Scopes and Objectives

Previous experiments pointed out R-phycoerythrin an interesting protein, taking into account some of their properties such as, high solubility in water, stability towards temperature, pH and time of storage [18], good optical and spectroscopic properties [73], and its bioactivity [7]. This protein also provides the possibility of numerous applications in various sectors namely in the food, pharmaceutical, cosmetic, and textile industries, and also in clinical medicine, diagnosis, and biomedical research [9].

Regarding the importance of R-phycoerythrin and the new differentiated domain of the regional program *CRER 2020* and the European program *Horizon 2020* centered in sea and marine resources as a sustainable biomass [1], the aim of this work is to use an abundant sea biomass in order to extract the proteinic pigments, and in particular R-phycoerythrin, considered the biomolecules of high added-value. These pigments, with particular focus in the R-phycoerythrin, will be extracted from the red macroalgae *Gracilaria vermiculophylla*, taking into account the search and application of alternative extraction technologies based on ILs. This macroalga was chosen for its high protein content [5], principally R-phycoerythrin [7]. In this context, this work will be divided in three major tasks as described below:

Task i) determination of an analytical method for the R-phycoerythrin quantification;

Task ii) application of different ILs on the extraction of R-phycoerythrin from the fresh solid biomass, avoiding drying costs and taking advantage the high water content of the macroalgae. In this task, the objective is to apply various ILs aqueous

solutions in the solid-liquid extraction of R-phycoerythrin aiming at the creation of crude extracts rich in the proteinic pigments;

Task iii) optimization of the main parameters of the extraction process, namely the time of extraction, the solid-liquid ratio, the pH, and the concentration of salt present in the buffer aqueous solution.

2. EXPERIMENTAL SECTION

In this section, methodologies are described regarding the optimization of a conventional methodology and the development of an alternative and more efficient methodology for the extraction of phycobiliproteins and R-phycoerythrin from the red macroalga *Gracilaria vermiculophylla.*

2.1 Materials and Methods

2.1.1 Materials

2.1.1.1 Macroalga

Fresh *Gracilaria vermiculophylla* (**Figure 12**) was collected in a growing environment of aquaculture (40.60°N and -8.67°E) in October 2014, February, April, June, July, and October 2015. It was kindly provided by ALGAplus Ltda, a company specialized in the production of marine macroalgae, located in Aveiro, Portugal. After the harvesting of the macroalgae, the samples were cleaned and washed with fresh and distilled water at least 3 times. Algae were weighted and then stored in a freezer at -20ºC until utilization. Dried *Gracilaria vermiculophylla* was also provided by ALGAplus and stored in a dried recipient in the absence of light for further studies.

Figure 12. Photography of the fresh *Gracilaria vermiculophylla* collected in October in the ALGAplus tanks.

2.1.1.2 Chemicals

The components of the sodium phosphate buffer (Na_2HPO_4/NaH_2PO_4), sodium phosphate dibasic heptahydrate, $Na_2HPO_4 \cdot 7H_2O$ (≥ 98 wt%), and sodium phosphate monobasic, NaH_2PO_4 (≥ 99 wt%), were purchased from Sigma-Aldrich® and Panreac, respectively.

Bovine serum Albumin was acquired from Acros Organics and purified R-phycoerythrin was purchased from Sigma-Aldrich®.

The series of 1-alkyl-3-methylimidazolium chloride ILs [$C_n$mim]Cl, including the 1-ethyl-3-methylimidazolium chloride, [C_2mim]Cl (98 wt%), 1-butyl-3-methylimidazolium chloride, [C_4mim]Cl (99 wt%), 1-hexyl-3-methylimidazolium chloride, [C_6mim]Cl (98 wt%), 1-methyl-3-octylimidazolium chloride, [C_8mim]Cl (99 wt%), 1-decyl-3-methylimidazolium chloride, [C_{10}mim]Cl (98 wt%), 1-dodecyl-3-methylimidazolium chloride, [C_{12}mim]Cl (>98 wt%), 1-methyl-3-tetradecylimidazolium chloride, [C_{14}mim]Cl (98 wt%), and other imidazolium-based ILs, including 1-ethyl-3-methylimidazolium acetate, [C_2mim][CH_3CO_2] (98 wt%), 1-butyl-3-methylimidazolium dicyanamide, [C_4mim][$N(CN)_2$] (98 wt%), 1-butyl-3-methylimidazolium trifluoromethanesulfonate, [C_4mim][CF_3SO_3] (99 wt%), 1-butyl-3-methylimidazolium acetate, [C_4mim][CH_3CO_2] (98 wt%), 1-butyl-3-methylimidazolium dimethylphosphate, [C_4mim][DMP] (>98 wt%), 1-butyl-3-methylimidazolium tosylate, [C_4mim][Tos] (98 wt%), 1-butyl-3-methylimidazolium methanosulfonate, [C_4mim][CH_3SO_3] (99 wt%), 1-butyl-3-methylimidazolium thiocyanate, [C_4mim][SCN] (98 wt%), 1-butyl-3-methylimidazolium trifluoroacetate, [C_4mim][CF_3CO_2] (97 wt%), 1-ethylimidazolium acetate, [C_2im][CH_3CO_2] (97 wt%), were acquired from IoLiTec (Ionic Liquids Technologies, Germany) as well as the remaining nitrogen-based cyclic ILs, 1-butyl-1-methylpiperidinium chloride, [C_4mpip]Cl (99 wt%), 1-butyl-1-methylpyrrolidinium acetate, [C_4mpyrr][CH_3CO_2] (97 wt%) and 1-butyl-1-methylpyrrolidinium chloride, [C_4mpyrr]Cl (99 wt%), 1-butyl-3-methylpyridinium chloride, [C_4mpyr]Cl (98 wt%), choline acetate, [Ch]Ac (>99 wt%). The tetrabutylammonium chloride, [$N_{4,4,4,4}$]Cl (97 wt%) and the cholinium chloride, [Ch]Cl (99 wt)% were purchased from Sigma-Aldrich®. Tetrabutylphosphonium chloride, [$P_{4,4,4,4}$]Cl, was kindly provided by Cytec and was first dried before use. The chemical structures of all ILs and salts used are depicted in **Figure 13**.

The sodium salts used in this work, namely sodium acetate, $NaCH_3CO_2$, and sodium dicyanamide, $NaN(CN)_2$ (96 wt%), were acquired from BDH Prolabo and Alfa Aesar, respectively. The water used was double distilled, passed by a reverse osmosis system, and further treated with a Milli-Q plus 185 water purification apparatus.

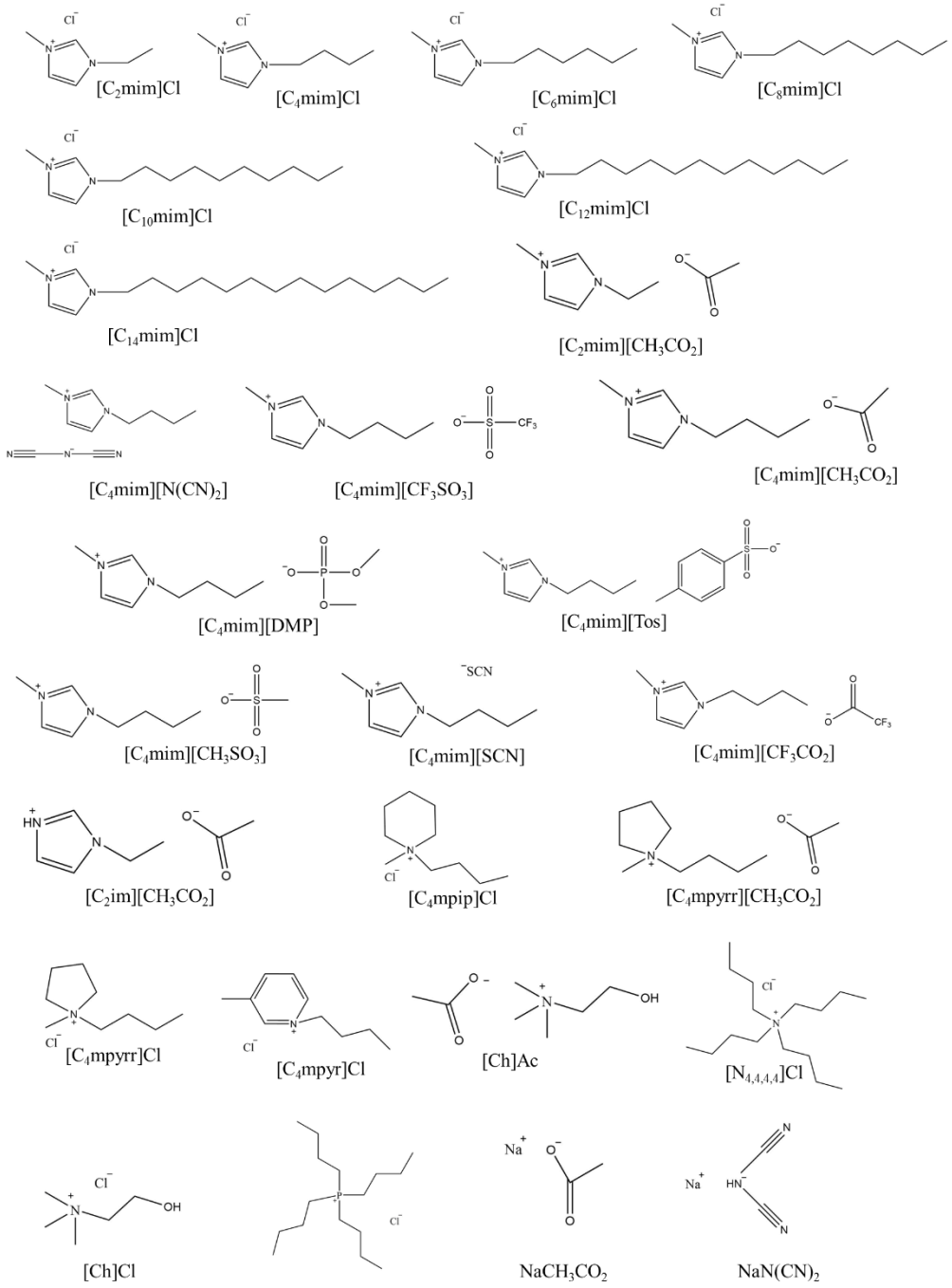

Figure 13. Chemical structure of the ILs and salts used.

2.1.2 Experimental Methodologies

2.1.2.1 R-phycoerythrin Conventional Extraction

The procedure adopted was based on a conventional methodology proposed by Liu and collaborators [43] used to extract R-phycoerythrin from the red macroalgae *Polysiphonia urceolata*, using a common sodium phosphate buffer. Frozen red

macroalgae samples were firstly grounded in liquid nitrogen and homogenized in 20 mM sodium phosphate buffer (pH 7) at room temperature, in an incubator shaker (IKA KS 4000 ic control) and protected from the light exposure (**Figure 14**). In the end of the extraction, the red solution was filtered and, subsequently, the filtrate originated was centrifuged in a Thermo Scientific Heraeus Megafuge 16 R Centrifuge at 5000 rpm for 30 minutes at 4ºC. The resultant pellet was discarded while a phycoerythrin-containing red supernatant was collected and the absorption spectra were determined in the interval between 200-700 nm in a UV-Vis microplate reader (Synergy HT microplate reader – BioTek). The quantification of R-phycoerythrin and total proteins in the extracts was made according to calibration curves (**Appendix A**: **Figure A1** and **Figure A2**, respectively), previously determined in the same UV-Vis equipment. Results are shown in terms of concentration of phycobiliproteins instead of concentration of R-phycoerythrin since the extracts are not purified and the absorption spectra of R-phycoerythrin may have the contribution of other phycobiliproteins.

Different conditions were tested in order to optimize the extraction process, namely the time of extraction and the solid-liquid ratio, once at a time.

Figure 14. Shacker used in the extraction experimental procedure.

2.1.2.2 R-phycoerythrin Alternative Extraction

A very similar procedure of extraction was initially used for the development of an alternative methodology, being the optimal conditions found for the conventional extraction applied. At this time, it was tested the effect of different ILs composed of

different anions and cations in aqueous solution for dried and fresh samples of *G. vermiculophylla* previously grounded. The solutions of ILs were prepared in 20 mM sodium phosphate buffer (pH 7) in order to reduce the occurrence of significant changes on the pH values of the solutions. However, since big changes of pH values occurred, pH was adjusted to approximately 7 with a NaOH (1 M) solution.

2.1.2.3 Surface Response Methodology

After the process optimization regarding the screening of various ILs aqueous solutions as solvents, a surface response was performed. The Surface Response Methodology (SRM) is a collection of mathematical and statistical techniques used to explore the relationship among several variables in an experiment in order to find, in this case, the optimal conditions of extraction for various conditions of the process and at the same time. The quantitative data from a proper experimental design is used to establish a mathematical model, reducing avoidable costs [74]. Since this experiment involves the combination of two or more factors, it was used a factorial design response surface. In this model, it is done a factorial study with two levels and k factors (independent variables). The number of runs of each experiment is given by the following equation:

$$2^k + 2k + Cx, \qquad 1)$$

being 2^k the number of factorial runs, $2k$ the number of axial runs, and Cx an random number of repetitions of the central point, which is expectable to be closer the best extraction conditions. It is very important to have several runs for the central point in order to know the residual plot and, consequently, the standard deviation and the repeatability quality of the experiment. In the other hand, the axial points are added to adjust the experiment. Given the conditions and the number of independent variables to be studied, it was chosen a design called Central Composite Rotatable Design, being the axial points calculated according to the α value,

$$\alpha = (2^k)^{\frac{1}{4}}, \qquad 2)$$

where ± α is the distance between the central and axial points [75].

Three independent variables were tested, namely the concentration of salt in the buffer solution, the pH of the solvent/system, and the solid-liquid ratio. Looking back to equation 1), and considering k =3 with a total of 20 extractions, it was performed 8

extractions for factorial points, 6 for axial points, and 6 repeats of the central point. Moreover, for a k=3 and considering the equation 2), α adopt a value of 1.68 [75].

The McIlvaine buffer was used in the extraction experiments as solvent and it was prepared according T. C. McIlvaine (1921) [76]. The salt concentration for the central point chosen was 0.5 M in other to embrace a large range of concentrations, according to the maximum solubility of Na_2PO_4, the salt used in the preparation of this particular buffer. Therefore, the solid liquid ratio (SLR) chosen for the central point is 0.7 and it is in accordance the previous assays of this work and with several published works (**Table 2**). In **Table 3** the central point and the calculated factorial and axial points are identified.

Table 3. Identification of central (0), factorial (± 1), and axial (± 1.68) points of a surface response with three independent variables.

	Axial Point **-1.68**	Factorial Point **-1**	Central Point **0**	Factorial Point **+1**	Axial Point **+1.68**
[Salt] (M)	0.2	0.3	0.5	0.7	0.8
pH	4.98	5.80	7.00	8.20	9.02
SLR	0.53	0.60	0.70	0.80	0.87

Once found all points to be tested (**Table 3**), and considering the design matrix (**Table 4**) result of an established mathematical model [75], the applied conditions of extraction were found for all three variables in the 20 extractions performed (**Table 4**).

Table 4. Design Matrix and decoded conditions for the surface response design for a 2^3 experiment.

Run	**Design Matrix**			**Decoded conditions**		
	[salt]	**pH**	**SLR**	**[salt]**	**pH**	**SLR**
1	-1	-1	-1	0.3	5.80	0.60
2	1	-1	-1	0.7	5.80	0.60
3	-1	1	-1	0.3	8.20	0.60
4	1	1	-1	0.7	8.20	0.60
5	-1	-1	1	0.3	5.80	0.80
6	1	-1	1	0.7	5.80	0.80
7	-1	1	1	0.3	8.20	0.80
8	1	1	1	0.7	8.20	0.80

9	-1.68	0	0	0.2	7.00	0.70
10	1.68	0	0	0.8	7.00	0.70
11	0	-1.68	0	0.5	5.00	0.70
12	0	1.68	0	0.5	9.02	0.70
13	0	0	-1.68	0.5	7.00	0.53
14	0	0	1.68	0.5	7.00	0.87
15	0	0	0	0.5	7.00	0.70
16	0	0	0	0.5	7.00	0.70
17	0	0	0	0.5	7.00	0.70
18	0	0	0	0.5	7.00	0.70
19	0	0	0	0.5	7.00	0.70
20	0	0	0	0.5	7.00	0.70

The analysis of the surface response results was made using the software STATISTICA 8.0 of Statsoft©.

2.1.2.4 Scanning Electron Microscopy (SEM)

About 1 mg of freeze dried samples was deposited on carbon conducting tape and coated with carbon. Scanning electron microscopy (SEM) images were obtained using a field-emission gun SEM Hitachi SU70 microscope operated at 15 kV and equipped with an energy-dispersive X-ray (EDX) spectroscopy accessory (EDX detector: Bruker AXS; Software: Quantax).

2.1.2.5 Detection of R-phycoerythrin and contaminants on a 2-D gel electrophoresis

The protein profile of different crude extracts collected was investigated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). All samples were diluted so the amount of protein in each gel lane was about 0.5 µg. This amount was predicted taking into account the UV quantification of the total amount of proteins in each phase. The electrophoresis was prepared on polyacrylamide gels (stacking: 4% and resolving: 20%) with a running buffer consisting of 250 mM of Tris HCl, 1.92 M of glycine, and 1% of SDS. The proteins were stained with the usual staining procedure [Coomassie Brilliant Blue G-250 0.1% (w/v), methanol 50% (v/v), acetic 7% (v/v), and water 42.9% (v/v)] in an orbital shaker, at moderate speed, during 2-3 hours and at

room temperature. The gels were distained in a solution containing acetic acid 7% (v/v), methanol 20% (v/v), and water 73% (v/v) in an orbital shaker at a moderate speed (± 60 rpm) during 3-4 hours at room temperature. SDS-PAGE Molecular Weight Standards, Marker molecular weight full-range (VWR), were used as protein standards. All gels were analyzed using the Image Lab 3.0 (BIO-RAD) analysis tool.

3. RESULTS AND DISCUSSION

The main objective of this work was the development of an alternative and more efficient extraction process to recover phycobiliproteins (and in particular R-phycoerythrin) from a red macroalgae using ILs as the main solvent. This non-conventional process of solid-liquid extraction was defined by the use of different aqueous solutions of ILs, thus considering the study of different families and chemical features. In this context, the main focus of this work will be, by applying ILs, the development of a one-step extraction approach to selectively recover phycobiliproteins.
As a biological material, the macroalgae biochemical composition can greatly vary according to the conditions previously exposed, namely seasonality and growth conditions. However, being the compound of interest a photosynthetic compound, one of the major influencing factors is the algae exposure to the natural light. It means that it is expectable that months with higher sunlight intensity promote an increase of chlorophyll and a decrease of the R-phycoerythrin content. These differences in R-phycoerythrin and chlorophyll content can be noticed directly by observation of the algae colour when harvested.
Due to the large water content of the macroalgae, at circa of 84% of water in *Gracilaria vermiculophylla*, a previous treatment in needed to correctly handle with this biomass. The same biomass treatment was followed: the fresh alga was weighted and stored in the freezer for further utilization, after defrosted. It should be highlighted that each sample defrosted was used and the remaining biomass was always discarded, since a second storage in the freezer changes completely the compounds of interest, the phycobiliproteins, causing their denaturation.
During this work there was a need to harvest *Gracilaria vermiculophylla* several times. In order to maintain the coherence of the results when applying the ILs, a comparison with the conventional extraction process was done (use of aqueous solutions of sodium phosphate buffer (20 mM), at pH 7) considering each IL, process condition and batch of macroalga investigated.

3.1. Conventional Extraction of R-phycoerythrin from fresh *Gracilaria vermiculophylla* using a phosphate buffer solution

The conventional methodology described in **Section 2.1.2.1** from this work was adapted from [43] uses aqueous solutions of a sodium phosphate buffer as solvent. The alternative methodology, under development in this work, is based on the use of aqueous solutions of different ILs, aiming at the extraction of phycobiliproteins from the fresh macroalga, but also focusing the selectivity of the solid-liquid extraction process, by describing a methodology capable to extract and at the same time, separate phycobiliproteins and chlorophylls, that together with the other classes of proteins (these without pigmentation properties) are considered as the main contaminants.

The conventional methodology was partially optimized, considering the time of extraction and solid-liquid ratio conditions, since this optimization will be also useful for the selection of the starting conditions of the alternative process.

3.1.1 Time of Extraction

The conventional methodology was used as benchmark and also to determine the total amount of *i)* phycobiliproteins and *ii)* total proteins (main contaminants) possible to be recovered from the biomass in just one step of extraction using aqueous solutions of a phosphate buffer. Thus, a kinetic study was carried out during 2 hours and considering a solid-liquid ratio of 0.625 (mass of fresh biomass/volume of solvent), being the results depicted in **Figure 15**.

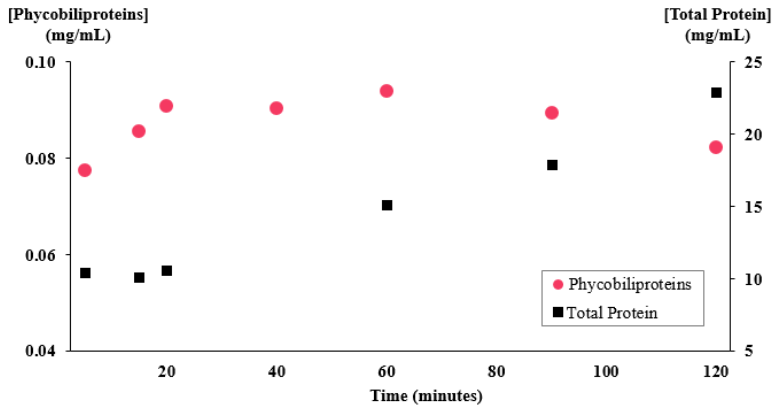

Figure 15. Maximum concentration of phycobiliproteins and total proteins extracted from the macroalga during 2 hours of extraction.

According to **Figure 15**, it is clear the general difference in both phycobiliproteins and total proteins content, being the latest the highest. Moreover, it is evident that the phycobiliproteins content of the extract reaches its maximum after 20 minutes, remaining constant until the end of the kinetic study. At the same time, the concentration of contaminant proteins increases significantly through time, starting at the 20 minutes. These results seem to indicate that R-phycoerythrin is firstly extracted due to its location in the external part of phycobilisomes (**Figure 4**). In this sense, trying to extract as much as possible the phycobiliproteins, avoiding as much as possible the extraction of the contaminant proteins, the time of extraction of 20 minutes was adopted further in this work.

3.1.2 Solid-liquid Ratio

Due to the importance attributed to the solid-liquid ratio in a very recent work focused in the extraction of carotenoids from brown macroalgae [77], and once fixed the time of extraction of 20 minutes, the effect of the solid-liquid ratio was investigated in the proteinic pigments extraction, considering the range of 0.1 to 1.0 (fresh weight of macroalga/volume of solvent) for macroalgae samples harvested in different months (**Figure 16**).

Firstly, by observation of **Figure 16** is clear the higher protein content of the macroalga harvested in February in comparison with the one harvested in October, probably due to the low sunlight intensity in winter months. Paying attention to the algal biomass harvested in October, it is possible to notice an increase of the total proteins content until a solid-liquid ratio of 0.6, thus remaining practically constant for the higher solid-liquid ratios. On the contrary, for the samples harvested in February, it is not possible to observe the same behavior, since the protein content increases in the entire range of solid-liquid ratios studied. These results are good indicators, because in a conclusive way it is possible to improve the extraction. If using a small volume of solvent the process is cheaper, also the experimental restrictions imposed by very high solid-liquid ratios are avoided, allowing thus the standardization of the solid-liquid ratio at 0.7, for which was obtained extracts of 0.28±0.08 and 36.5±6 mg/mL for phycobiliproteins and total proteins, respectively, for alga harvested in February.

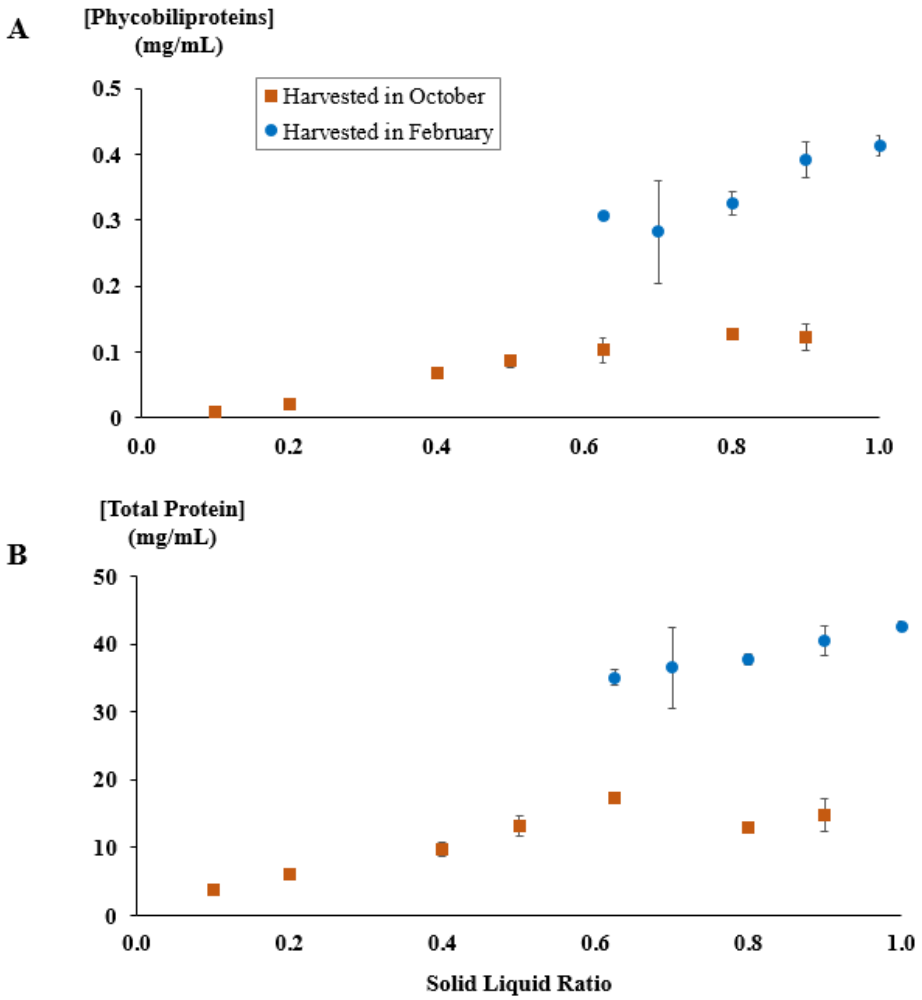

Figure 16. Variation of the **(A)** phycobiliproteins and **(B)** total protein content extracted from the macroalga, considering the use of different solid-liquid ratios under study.

3.2. Alternative Extraction of Phycobiliproteins

As previously discussed, the conventional methodology applied on the extraction of phycobiliproteins from the *Gracilaria vermyculophylla* macroalga is not very selective, i.e. if this method extracts phycobiliproteins from the biomass, the amount of contaminant proteins is significantly higher, which means that the cost associated with the next task, the purification of the phycobiliproteins will be much higher, due to the abusive quantity of contaminants. It was in this context that this work has made an attempt to investigate an alternative methodology based on ILs to extract as much as possible the phycobiliproteins (and R-phycoerythrin), while lowering the amount of contaminants extracted. Therefore, a screening of alternative solvents, namely ILs, was

made in order to understand their influence on the extraction of the target proteins, being these results compared with the experimental data originated from the use of the sodium phosphate buffer. Due to the lack of knowledge about the use of ILs extracting these proteins, the starting concentration was fixed at 1 M. Considering the need to identify the most appropriate ILs, and due to the large number of tests being planned, the entire step of optimization of this work was performed with dried and fresh biomass. In the case of dried biomass, it was firstly hydrated until saturation and then the experimental tests were done taking into account the experimental conditions optimized for the conventional methodology, being the solid-liquid ratio the exception. This parameter was fixed at 0.7 but this time considering the use of dried biomass (dried biomass/volume of solvent).

The ILs screen started with the extraction process being done with various imidazolium-based ILs and aiming to test the influence of the alkyl chain length, but also several cation families and anions were investigated.

3.2.1 Extraction of Phycobiliproteins from dried *Gracilaria vermiculophylla*

3.2.1.1 Alkyl chain length

In a first attempt, the effect of the alkyl chain length of imidazolium-based ILs was tested. Thus, series of 1-alkyl-3-methylimidazolium chloride ILs were used, namely [C_2mim]Cl, [C_4mim]Cl, [C_6mim]Cl, [C_8mim]Cl, [C_{10}mim], [C_{12}mim]Cl, and [C_{14}mim]Cl. The crude aqueous extracts obtained from each extraction process as well as the absorbance spectra collected are depicted in **Figure 17**.

The careful analysis of these experimental results is crucial to understand the impact that such ionic solvents can have, when in contact with the dried algal biomass. The aspect of the various extracts is very different when all the [$C_n$mim]Cl-based ILs are analyzed. This gives instantaneously the idea that their biochemical profile is different and thus, different bioactive compounds in distinct concentrations are being removed from the solid biomass. If for shorter alkyl chains, namely [C_2mim]Cl, [C_4mim]Cl, and [C_6mim]Cl, the crude aqueous extracts with their pink color are similar to the one obtained by the sodium phosphate buffer; for longer alkyl chains, namely [C_8mim]Cl, [C_{10}mim], [C_{12}mim]Cl, and [C_{14}mim]Cl, the colors of the crude aqueous extracts obtained are yellow or green.

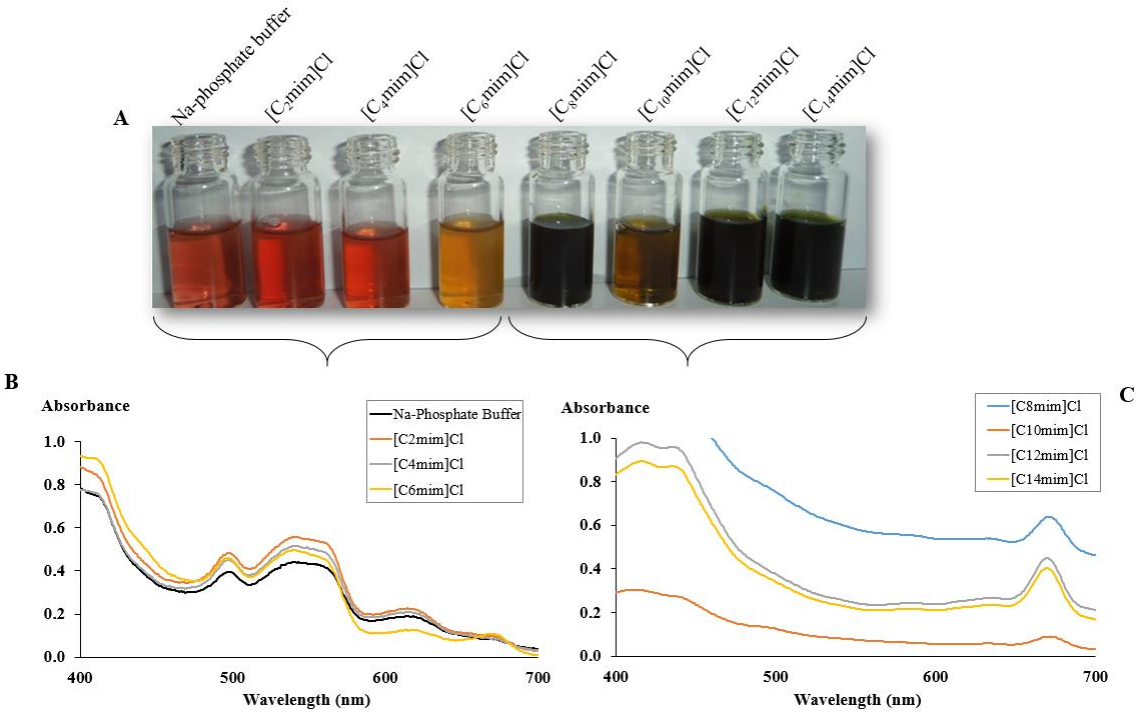

Figure 17. Main results obtained when different [$C_n$mim]Cl-based ILs are used: **(A)** photography of the various crude extracts obtained from the extraction process; **(B)** absorption spectra of crude extracts obtained by the used of the short alkyl chain-based [$C_n$mim]Cl ILs and **(C)** absorption spectra of crude extracts obtained by the used of the long alkyl chain-based [$C_n$mim]Cl ILs.

The different profiles achieved are related with the compounds preferentially extracted in each extract, which are different and dependent of the ILs used, thus being proved and justified by the absorbance spectra depicted in **Figure 17**B and C. If for the shorter alkyl chains, the spectra is evidencing the characteristic peaks of the phycobiliproteins, for the longer alkyl chains, the spectra is describing the absorbance peaks corresponding to carotenoids and chlorophylls [78]. These results can be explained by the hydrophobicity/hydrophilicity nature of the ILs. When more hydrophilic ILs are applied (*i.e.* ILs with shorter alkyl chains) the phycobiliproteins are preferentially extracted due to their higher hydrophilicity, on the contrary, when hydrophobic ILs are tested, the most hydrophobic bioactive compounds present in the biomass cells are preferentially extracted, meaning carotenoids and chlorophylls (present in the biochemical composition of red macroalgae as an intervenient in the light energy transfer pathway in phycobilisomes).

Moreover, the results found for the long alkyl chain ILs can also be explained by their capacity to act as surfactants, due to their amphiphilic behavior [79], if they have

charged hydrophilic head groups, they also have an hydrophobic 'tail' domain, thus allowing their auto-aggregation in water and the consequent formation of micelles [79]. In fact, the higher affinity of carotenoids [77] and chlorophylls (data unpublished) for micelles was already shown. Behind the carotenoids and chlorophylls extracted from the macroalga by the ILs with longer alkyl chains, other compounds namely lipids and other are being removed, thus being interfering with the baseline of the spectra, making impossible any attempt to quantify the target proteins. In this sense, the ILs with the shorter alkyl chains were the ones selected for further studies.

3.3.1.2 Cation Core and Anion Moiety effect

Considering the advantageous extraction performance for the phycobiliproteins demonstrated by the shorter alkyl chains, the study of different families (two aromatic cations: imidazolium and pyridinium, two cyclic cations: pyrrolidinium, piperidinium and two acyclic cations: ammonium and cholinium) and anions was done taking into account ILs with alkyl chains no longer than 6 carbons. Contrarily to what was verified for the alkyl chain length effect, in this case, the extracts, have in general, similar aspects and colors, thus demonstrating their potential higher efficiency to extract the target proteins from the algal biomass, instead of contaminants (**Figure 18**).

Considering the concentration of phycobiliproteins determined for each one of the crude aqueous extracts presented in **Figure 18**A, the main conclusion was that the various ILs seems have not a significant influence on the phycobiliproteins extracted, even when compared with the experimental result found for the conventional method using the sodium phosphate buffer. Yet, in what concerns the conventional method, it seems that the extract here obtained is less fluorescent when compared with the aqueous extracts obtained for the fresh biomass. This behavior can be the consequence of *i)* the use of non-appropriate process conditions, *ii)* the saturation of the solvent inhibiting the complete extraction of phycobiliproteins or *iii)* the drying process applied to the macroalgae is significantly changing or irreversibly degrading the structure of phycobiliproteins, including R-phycoerythrin.

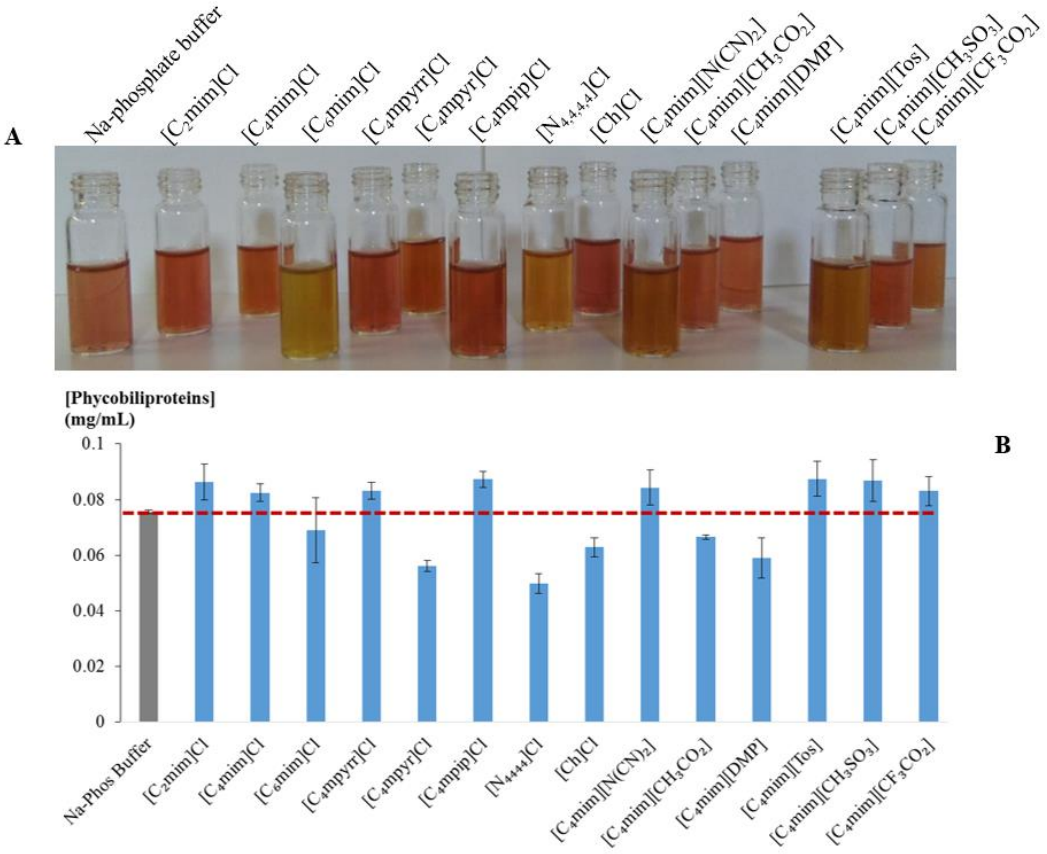

Figure 18. Main results obtained when different ILs are used: **(A)** photography of the various crude extracts obtained from the extraction process; **(B)** concentration of phycobiliproteins obtained in the crude extracts by the used of ILs with different cations and anions.

Trying to better understand the phenomenon behind the set of results obtained for the dried biomass, the cell structure of the fresh biomass samples was analyzed by SEM and compared with fresh samples, both grounded (**Figure 19**).

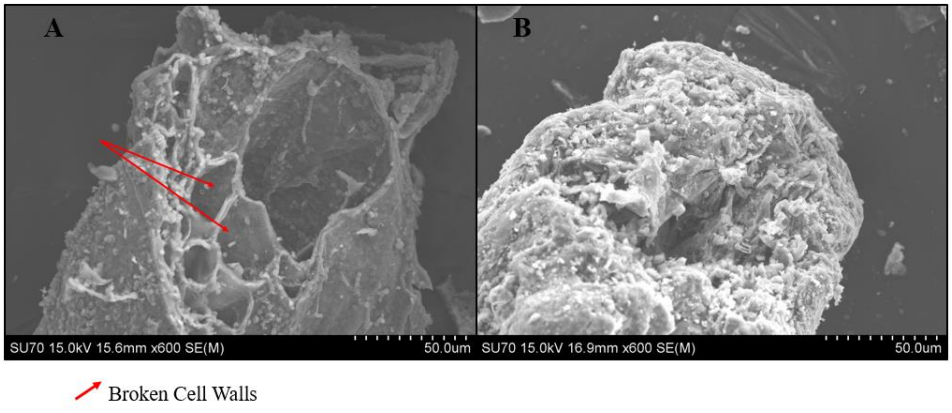

Figure 19. Scanning Electron Microscopy of the grounded **A:** fresh, and **B:** dried *Gracilaria vermiculophylla* biomass cells.

It is possible to see that, contrarily to what happens with the fresh biomass (**Figure 19**A), the dried macroalga did not suffer cell wall breakage during the grinding process (**Figure 19**B). This may be explained by the action of the cells' water content, which swells the cells, facilitating the damage of the biomass and thus enhancing the extraction of phycobiliproteins. Considering these results and others recently found [77], further studies were performed using the fresh macroalga.

3.2.2 Extraction of Phycobiliproteins from fresh *Gracilaria vermiculophylla*

3.2.2.1 Alkyl chain length effect

With the same propose, a set of different ILs were tested considering the extraction of phycobiliproteins from the fresh biomass. The crude aqueous extracts obtained as well as the absorbance spectra collected for each IL and from the phycobiliproteins and contaminant proteins extracted are depicted in **Figure 20**.

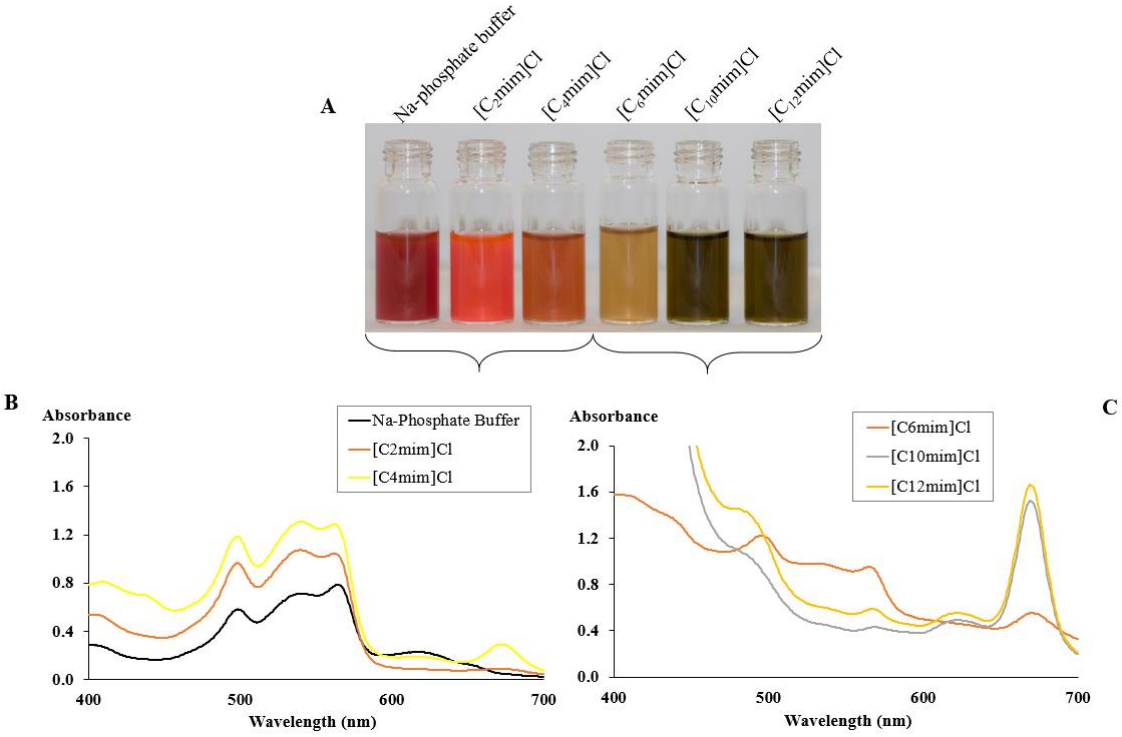

Figure 20. Main results obtained when different [$C_n$mim]Cl-based ILs are used: **(A)** Photography of the various crude extracts obtained from the extraction process; **(B)** absorption spectra of crude extracts obtained by the used of the short alkyl chain-based [$C_n$mim]Cl ILs and **(C)** absorption spectra of crude extracts obtained by the used of the long alkyl chain-based [$C_n$mim]Cl ILs.

By comparison with the results obtained for the dried biomass, the general conclusions remain. Smaller alkyl chains, namely [C_2mim]Cl and [C_4mim]Cl, promote the extraction of phycobiliproteins and R-phycoerythrin. However, in this case the use of [C_4mim]Cl seems to be able to extract a small amount of chlorophyll, as described by the presence of a small peak at 670 nm. For ILs with longer alkyl chains ([C_6mim]Cl, [C_{10}mim]Cl, and [C_{12}mim]Cl), again it is not possible to identify the characteristic peaks of phycobiliproteins and R-phycoerythrin. Once more, for the longer chains, the extraction of chlorophyll seems to become more relevant. As previously discussed, these results seem to support the same idea that ILs with longer alkyl chains act as surfactants, allowing preferentially the extraction of more hydrophobic molecules such as chlorophyll. Taking into consideration the set of results discussed, it is concluded that the use of different ILs in a sequential extraction process may be relevant for the R-phycoerythrin and chlorophyll extraction, enhancing thus the value and the potential markets of this biomass.

3.2.2.2 Cation effect

The effect of different cations with small alkyl chains (4 carbons) was investigated in the extraction of R-phycoerythrin. Apart from the previously studied [C_4mim]Cl, other aromatic cation was investigated, the [C_4mpyr]Cl, two non-aromatic cyclic cations, the [C_4mpyrr]Cl, and [C_4mpip]Cl, and three non-cyclic families, namely [Ch]Cl, [$N_{4,4,4,4}$]Cl, and [$P_{4,4,4,4}$]Cl. The extracts and absorption spectra obtained for the different families are shown in **Figure 21**.

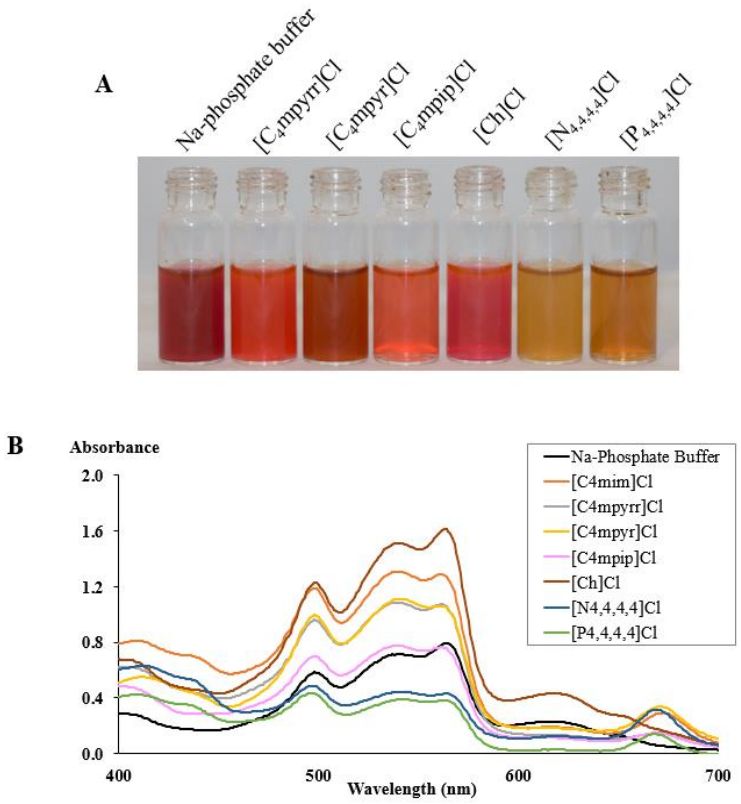

Figure 21. Main results obtained when different chloride-based ILs are used: **(A)** photography of the various crude aqueous extracts obtained from the extraction process; **(B)** absorption spectra of crude aqueous extracts obtained.

Looking for the main results achieved for the various cations, it seems that the phycobiliproteins (not only R-phycoerythrin but also, for some cases, R-phycocyanin and allophycocyanin, indicated by the maximum peak at 620 nm) were extracted (**Figure 21**B), which is proved not only by the colour of the crude aqueous extracts, but principally, by the absorbance spectra, regarding the wavelength range between 500 and 600 nm. Taking into account the spectra, it seems that the crescent order of capacity to extract the phycobiliproteins in just one step of extraction and without any further optimization is $[P_{4,4,4,4}]Cl$ < $[N_{4,4,4,4}]Cl$ < Na-phosphate buffer < $[C_4mpip]Cl$ ≈ $[C_4mpyrr]Cl$ < $[C_4mim]Cl$ < [Ch]Cl. The concentration of phycobiliproteins composing each one of the crude aqueous extracts is depicted in **Figure 22**.

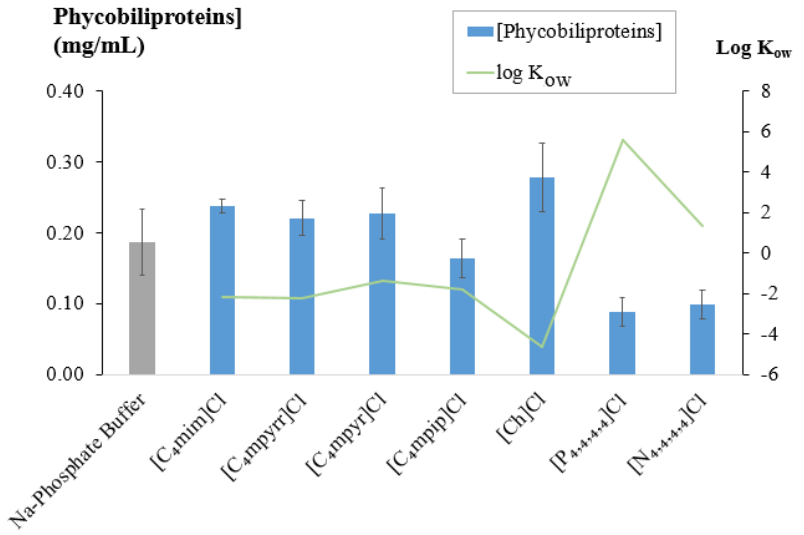

Figure 22. Phycobiliproteins concentration present in the crude aqueous extracts obtained by the use of different ILs as solvents. The logarithmic function of the octanol-water partition coefficients (log K_{ow}) is also represented. Values available in [80].

In general, the main results show that $[P_{4,4,4,4}]Cl$ and $[N_{4,4,4,4}]Cl$ seem to be the less effective in the extraction of phycobiliproteins due to their higher hydrophobic nature [81]. On the contrary, the more hydrophilic ILs seem to be the most effective, even when compared with the sodium phosphate buffer, in extracting the phycobiliproteins and, consequently the R-phycoerythrin from the fresh biomass.

The hydrophilic/hydrophobic balance of the ILs can be explained by the octanol-water partition coefficient (K_{ow}) [82], which represents the ratio between the concentration of a target compound between the 1-octanol and water. Thus, the log K_{ow} values of $[P_{4,4,4,4}]Cl$ and $[N_{4,4,4,4}]Cl$ are the highest (and the only positive values) in this set of ILs, which makes them the most hydrophobic. However, it should be highlighted that, despite the efficiency of some ILs extracting the phycobiliproteins, some also have the capacity to remove some of the chlorophyll content present in the biomass. This means that, despite the higher extraction of the target molecules, the extracts originated are more contaminate, and this issue needs to be taken into account in the step of purification.

3.2.2.3 Anion effect

The effect of different ILs' anions in the phycobiliproteins extraction was also contemplated in this work, being the main results depicted in **Figure 23**. The study of the anions effect was realized taking into account the 1-butyl-3-methylimidazolium cation and the following anions: chloride, dicyanamide, tosylate, dimethylphosphate, thiocyanate, trifluoromethanesulfonate, trifluoroacetate, methanosulfonate, and acetate. In addition to the image of the crude aqueous extracts, the phycobiliproteins content in each extract was quantified, as recorded in **Figure 23**B, as well as the β values [83], as a parameter representing the hydrophilic/hydrophobic character of the ILs when the anions are under study.

One more time, a diversity of different extracts, considering the color were originated from the use of distinct ILs based on different anions. By the analysis of **Figure 23**B, it is possible to see that Cl^-, $CH_3CO_2^-$, and $CH_3SO_3^-$ are the anions with higher capacity to extract the phycobiliproteins and, consequently, the R-phycoerythrin. On the other hand, $[N(CN)_2]^-$, $[SCN]^-$, and $[CF_3SO_3]^-$ are the worst anions extracting phycobiliproteins while at the same time are extracting chlorophylls (reason of the yellow/brown color of some aqueous extracts - **Figure 23**A). Again, these results can be explained by the hydrophobic/hydrophilic nature of each anion, described by the solvatochromic parameter β, which is describing the hydrogen-bond acceptance capacity [83,84]. In this context, the β value increase, augments the hydrogen-bond formation possibility, which seems to enhance the phycobiliproteins extraction.

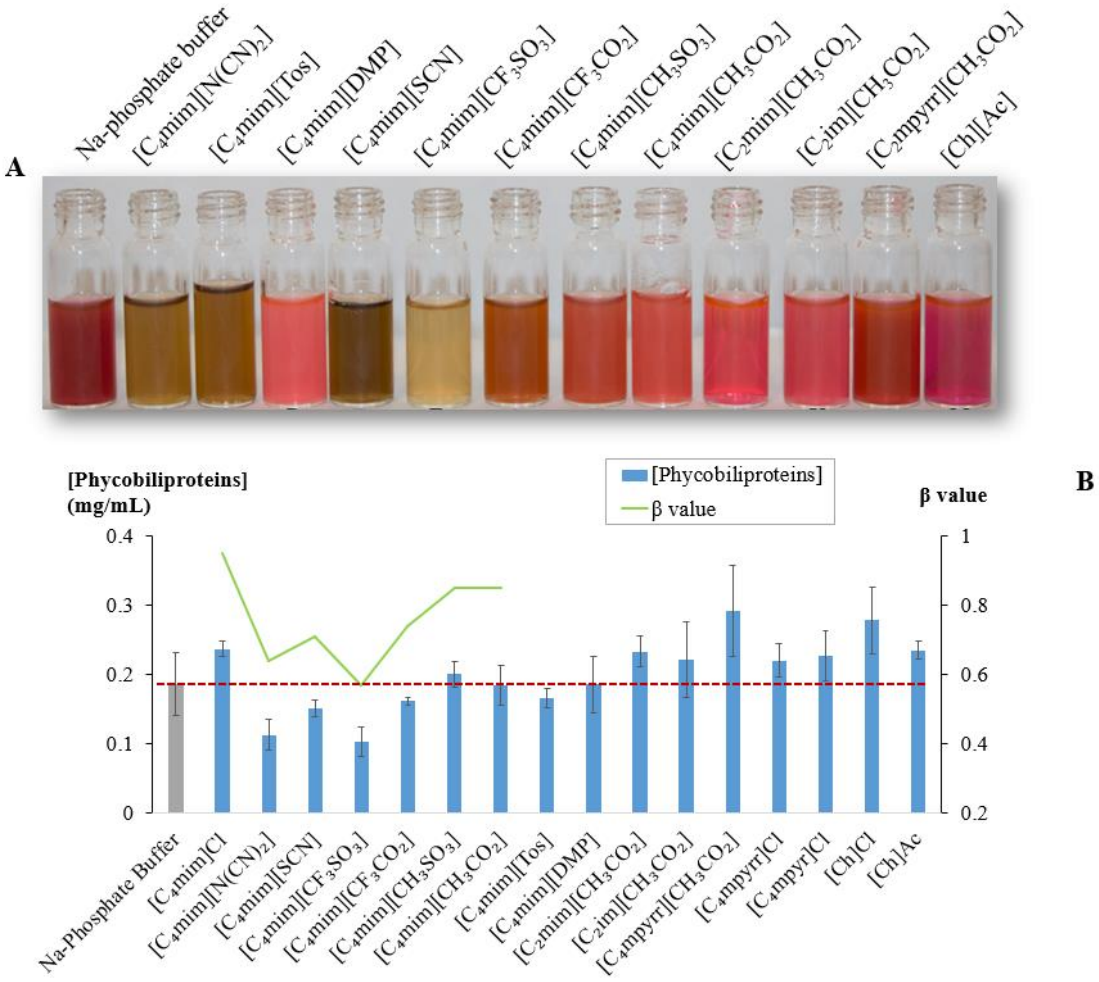

Figure 23. Main results obtained when different ILs with small alkyl chain are applied in the solid-liquid extraction: **(A)** photography of the various crude aqueous extracts obtained; **(B)** phycobiliproteins concentration in crude aqueous extracts obtained with ILs, and their respective β values [83].

3.2.2.4 Salts and mixtures of salts and ILs as solvents

Considering the high impact of some ILs and some anions in particular, the respective salts were also tested, since the success of these salts in the extraction mean lower costs of the process, which is clearly justified by the lower prices of common salts when compared with the respective ILs congeners. Thus, some common salts (sodium acetate, sodium dicyanamide) and equimolar mixtures of salts and ILs (sodium acetate + [C_2mim]Cl and sodium dicyanamide + [C_2mim]Cl) were tested. The aqueous crude extracts obtained are present in **Figure 24**A, being their respective absorption spectra depicted in **Figure 24**B.

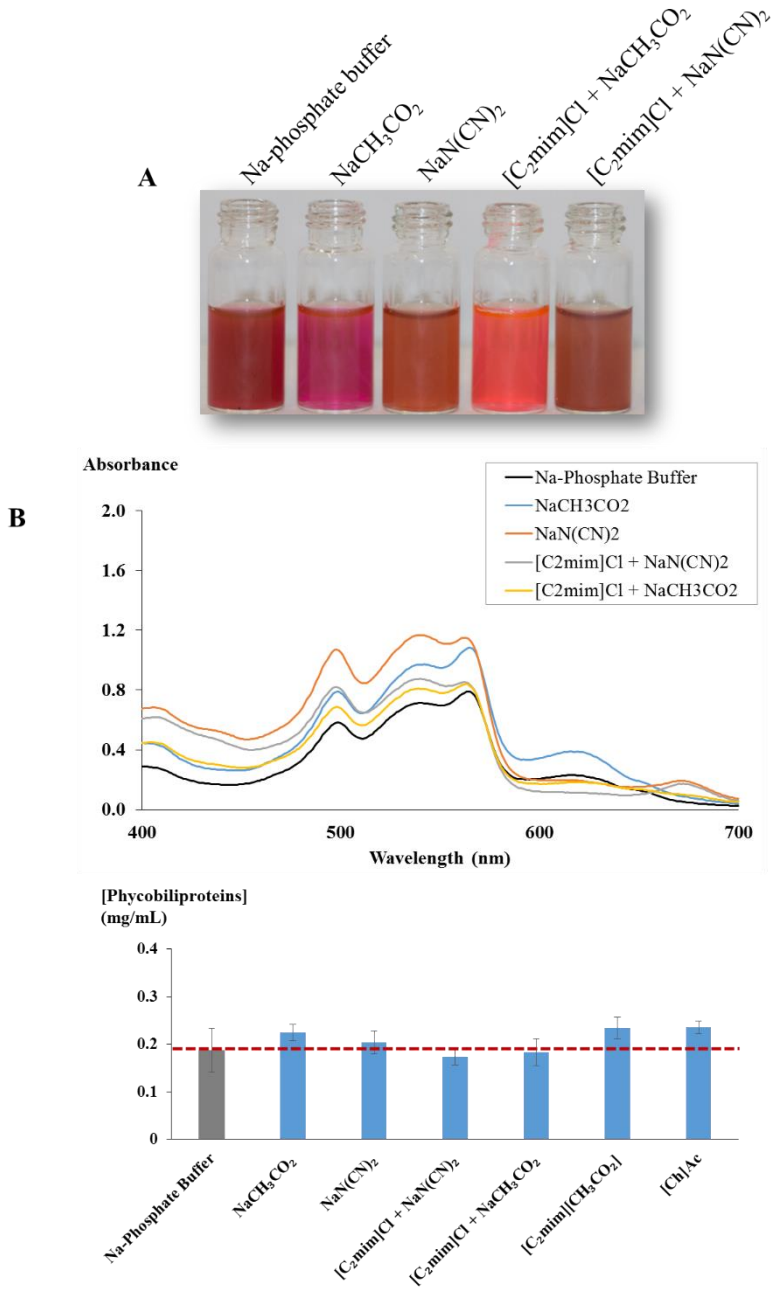

Figure 24. Main results obtained when different using salts and equimolar mixtures of salts and ILs are used: **(A)** photography of the various aqueous crude extracts obtained from the extraction process; **(B)** absorption spectra of aqueous crude extracts obtained; **(C)** concentration of phycobiliproteins quantified in the crude aqueous extracts.

The main results (absorption spectra) suggest that all solvents used, salts and equimolar mixtures of salts and ILs, were able to extract phycobiliproteins and R-phycoerythrin from the biomass, which is confirmed by the pink color and fluorescence of the aqueous extracts. Moreover, and yet by the careful analysis of the absorption spectra, it is

concluded that the sodium dycianamide and the equimolar mixture of sodium dycianamide + [C_2mim]Cl are also extracting some amounts of chlorophyll, here considered as contaminants. In the extracts obtained with [C_4mim][$N(CN)_2$] it was visible some color loss from pink to brown, which may be due to some degree of protein denaturation caused by the IL presence.

Once again, hydrophilic solvents such as the sodium phosphate buffer and the aqueous solution of $NaCH_3CO_2$ promoted the formation of extracts with a maximum peak closer to 620 nm, meaning that other phycobiliproteins, apart from R-phycoerythrin, were extracted. In the other hand, when $NaCH_3CO_2$ + [C_2mim]Cl aqueous solutions are tested, this maximum at 620 nm is not visible, making this solvent more selective for R-phycoerythrin. Analyzing the concentration of phycobiliproteins determined again for each solvent (**Figure 24**C), it is observed that as already visually concluded, there are no significant differences between the extracts.

3.3. Optimization of operational conditions

Firstly, a comparison about the effect of both sodium phosphate and McIlvaine buffers, using previously fixed conditions to extract phycobiliproteins (and R-Phycoerythrin) was assessed (**Figure 25**).

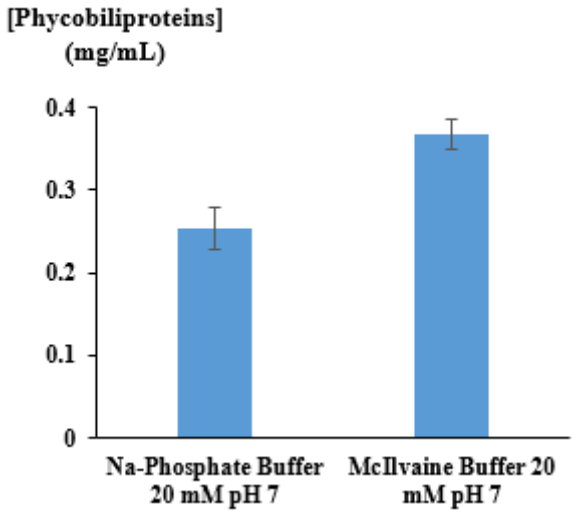

Figure 25. Concentration of phycobiliproteins extracted by the application of two distinct buffers.

It is possible to conclude that, apart from the easier manipulation of the pH for a wider range of values, the McIlvaine buffer allows a more efficient extraction of

phycobiliproteins (and R-phycoerythrin) in comparison with the sodium phosphate buffer. At this point, the McIlvaine buffer was chosen to allow the study of a wider range of pH values, since the pH is one of the most important conditions to be tested when the target products are proteins. Moreover, and after the proper selection of the ILs with higher capacity to extract the phycobiliproteins content of the macroalgae, the optimization of different process conditions was performed. Examples of these conditions are the solid-liquid ratio (SLR), the pH, and the concentration of salt composing the buffer used in this study ([salt]) tested by applying a Response Surface Methodology (RSM). This analytical methodology allows the investigation of different conditions at the same time and the determination of the relationship between the phycobiliproteins concentration extracted from the marine biomass (response variable) and the operational conditions of interest for the solid-liquid extraction process (independent variables). For the RSM, a 2^3 factorial planning study (3 factors and 2 levels) was executed (**Appendix B: Table B1**). SLR, pH and [salt] were the conditions optimized, being the experimental points used, the model equations, the phycobiliproteins' concentration experimentally and theoretically achieved using the correlation coefficients obtained in the statistical treatment, as well as all the statistical analyses.

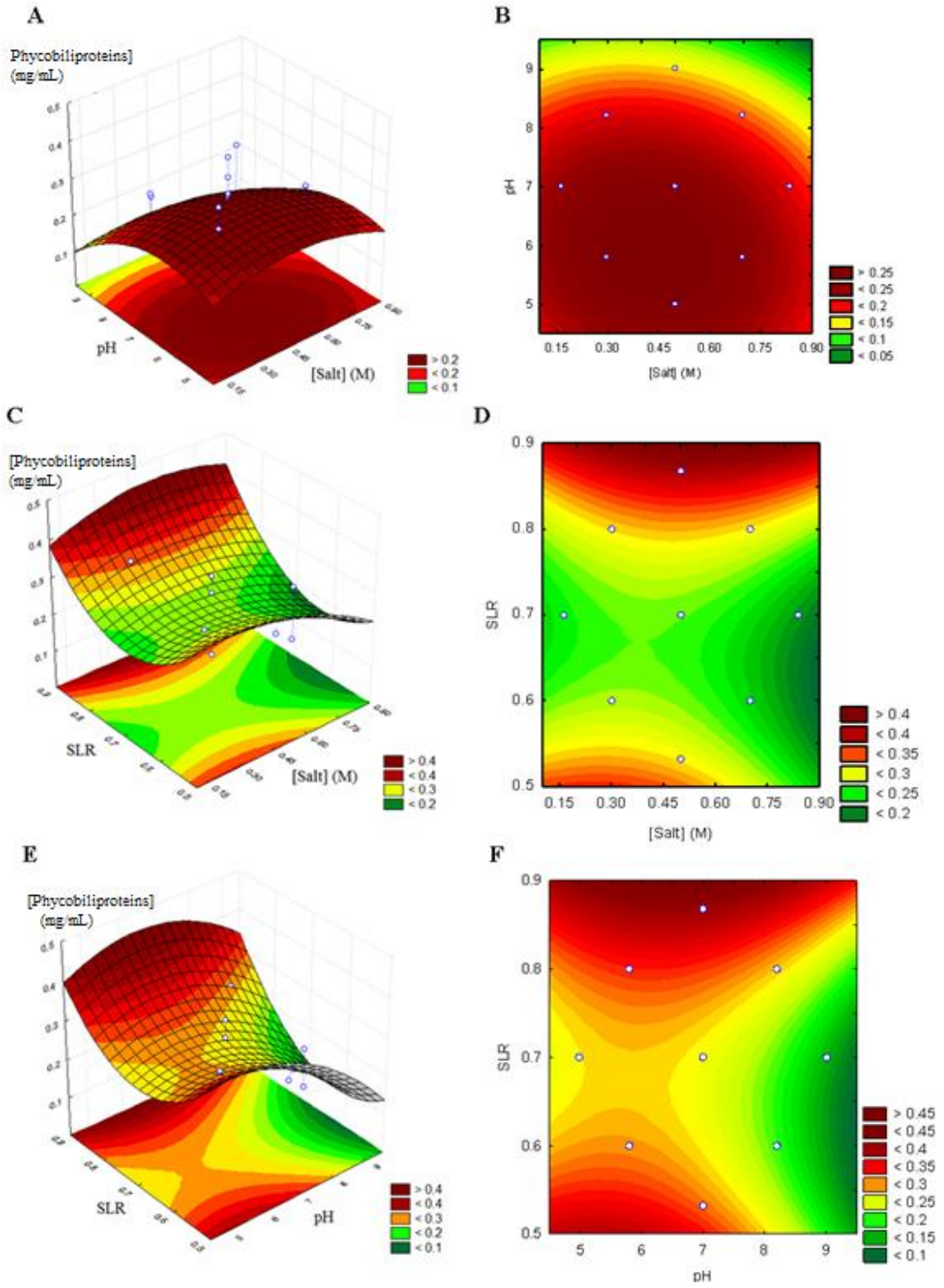

Figure 26. Response surface plots (left) and contour plots (right) on the phycobiliproteins concentration (mg/mL) with the combined effects of **(A** and **B)** pH and salt concentration (M); **(C** and **D)** solid-liquid ratio and salt concentration (M); and **(E** and **F)** solid-liquid ratio and pH of the solvent, using McIlvaine buffer as solvent.

The accuracy and the precision of the model equations can be validated by the comparison of the experimental and the predicted values of extraction yield under the chosen conditions. No significant differences (*p*-value = 0.05) were observed between the theoretical and experimental responses (**Figure B1**, and **Table B1**, **B2** and **B3**).

Figure 26 depicts the effect of the three variables studied in the concentration of phycobiliproteins extracted in a single-step of extraction. The main results presented in the response surface and contour plots seems to suggest that the most significant condition studied was the pH and SLR. Regarding the SLR, the results indicate that the higher the SLR, the higher the extraction of phycobiliproteins (**Figure 26**C and F). However, and as previously observed (**Section 3.1.2**), very high SLR hinder the extraction process itself, especially above 0.7, which means that this experimental restriction should be taken into account in further studies. The pH data indicates that it has a theoretical maximum near to 6, while the [salt] of the buffer has no significant effect (**Figure 26**A and B). This means that the salt present in solution is only important to maintain the buffer condition of the system, but it is not responsible for the potential negative effect on the cell disruption or by the increase in the solubility of the phycobiliproteins in water. These results are also proved by the Pareto's Diagram, represented by in **Figure 27**.

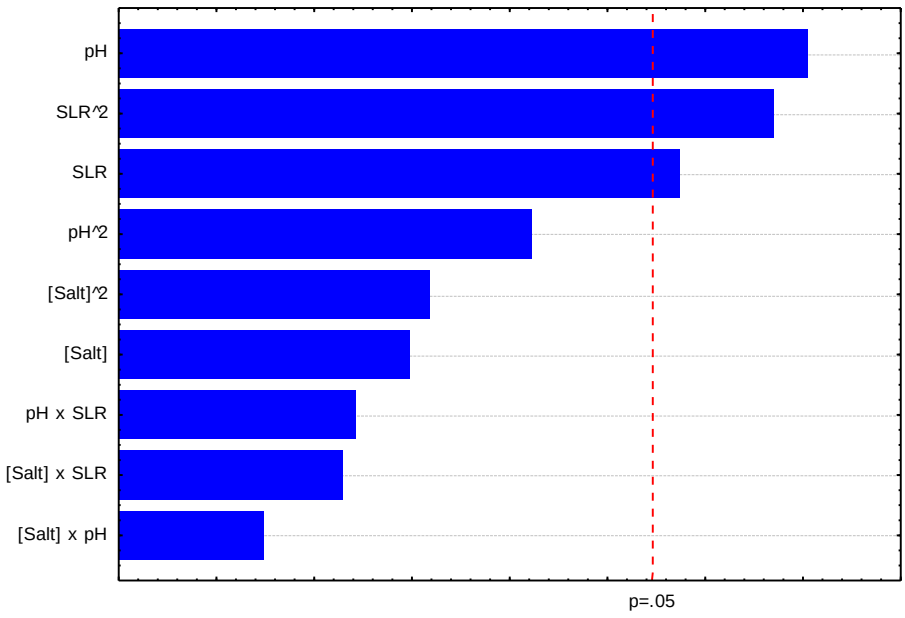

Figure 27. Pareto chart of standardized effects using a factorial design of 2^3, being phycobiliproteins concentration (mg/mL) variable.

3.3.1 Experimental validation of the optimum pH value

The surface response design previously analysed as called the attention mainly for the importance of the pH optimization. Since the compound to extract is a protein, it is expectable that R-phycoerythrin can suffer with pH variations, mainly because pH may induce conformational changes that can be dramatic for extreme pH values. Moreover, the change of pH solvent causes the change of the all environment, changing the non-covalent bonding between molecules, and therefore, influencing the extraction [85]. Munier et al. found that there is no conformational changes of R-phycoerythrin extracted from the red macroalga *Grateloupia turuturu* for pH values among 4 and 10 [18]. Different pH values were studied in the extraction of R-phycoerythrin from *Gracilaria vermiculophylla* and the absorption spectra of the extracts are in **Figure 28**.

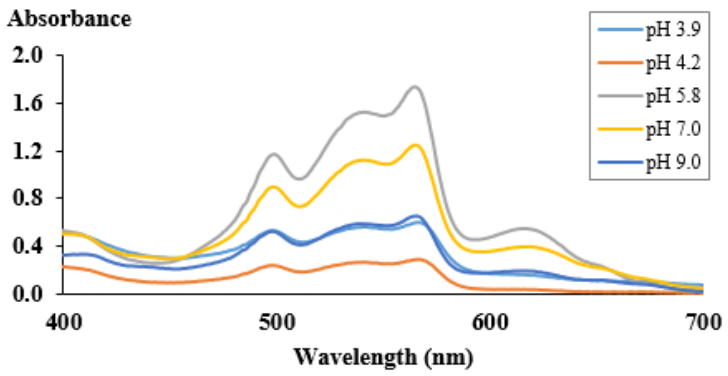

Figure 28. Absorption spectra of extracts from red macroalgae using as solvent McIlvaine buffer at different pH values.

By analysis of the absorption spectra, there are no evidences of structural changes in R-phycoerythrin at these studied pH values. Even for extreme pH values such as 3.9 and 9, R-phycoerythrin is still being extracted proving its stability at a wider range of pH. Focusing on the phycobiliproteins content of the extracts, **Figure 29** represents a study of the pH effect on their extraction, and its confrontation with the theoretical maximum given by the surface response design and the method validation.

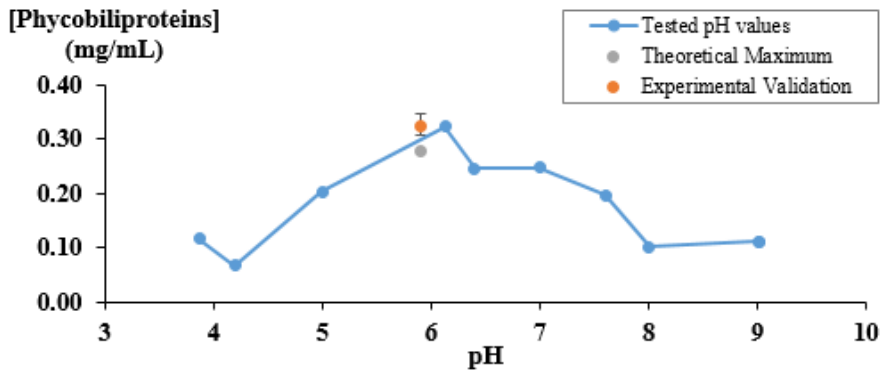

Figure 29. Effect of the pH of the solvent in the phycobiliproteins extraction: tested pH values, theoretical maximum by the SRM and experimental validation of theoretical data.

The blue line represents an expanded tendency, pointing out better extractions for pH between 5 and 7. For higher and lower pH, the solubility of the protein decreases. At pH 4.2, the isoelectric point of the R-phycoerythrin [86], the extraction achieved the lowest value because at this pH the protein is not charged [85].

Coming back to the analysis of the surface response, **Figure 26**A is given by the following equation:

$$[\text{Phycobiliproteins}]\ (\text{mg/mL}) = -0.313364794\ [\text{Salt}]^2 - 0.015431840\ [\text{Salt}]\ .\ \text{pH} + 0.362988\ [\text{Salt}] - 0.012899866\ \text{pH}^2 + 0.15855\ \text{pH} - 0.265556\ ,$$

which has its maximum of 0.28 mg/mL for a salt concentration of 0.43 M and a pH of 5.9 (point in grey in **Figure 29**). In order to claim the validity of the method for the optimal extraction given by SRM, the theoretical maximum was performed experimentally three times (point in orange in **Figure 29**). The orange point can easily be incorporated in **Figure 29**. Moreover, it is way near of the theoretical maximum, ensuring the success of the assay, and allowing the adoption of 5.9 as the optimum pH.

3.4 Biomass analysis by Scanning Electron Microscopy

In order to better understand the effect of the solvent in the extraction process, a structural analysis of the algae was performed by SEM (**Figure 30**) taking into account different scenarios of optimization. The biomass after extraction with: sodium phosphate buffer, McIlvaine buffer and [Ch]Cl was analyzed for its ability to extract

phycobiliproteins, while [C_4mim]Tos was chosen for its ability to extract simultaneously phycobiliproteins and chlorophyll, and [C_{12}mim]Cl for its capacity to extract just chlorophyll.

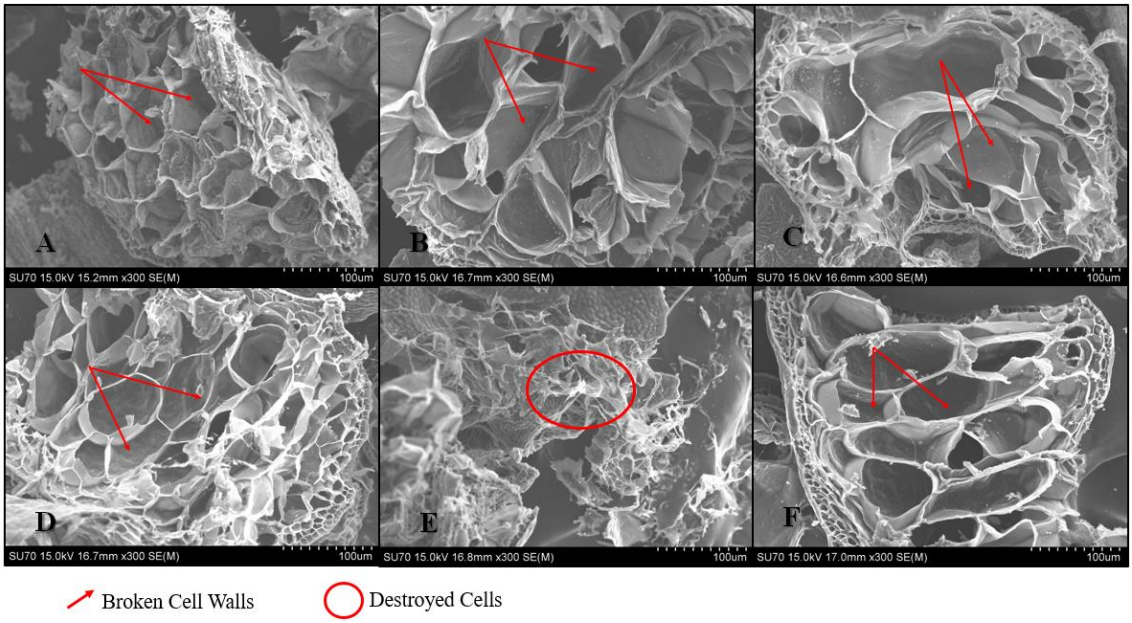

Figure 30. Scanning Electron Microscopy of the *Gracilaria vermiculophylla* biomass cells, being **(A)** grinded cells; **(B)** cells after extraction with sodium phosphate buffer; **(C)** cells after extraction with McIlvaine buffer; **(D)** cells after extraction with [Ch]Cl; **(E)** cells after extraction with [C_4mim]Tos; and **(F)** cells after extraction with [C_{12}mim]Cl.

No differences are noticed among the control (only grinded cells, without any contact with any solvent - **Figure 30**A) and the samples which were in contact with: sodium phosphate buffer (**Figure 30**B), McIlvaine buffer (**Figure 30**C), [Ch]Cl (**Figure 30**D), and [C_{12}mim]Cl (**Figure 30**F). It makes clear that the grinding step is a key point in the whole extraction process. It promotes the breaking of cell walls, helping the contact between the solvent and the phycobiliproteins and R-phycoerythrin. In the other hand, [C_4mim]Tos (**Figure 30**E) is promoting an additional cell damage, enhancing the cell content exposure to the solvent, and thus, eventually enhancing the extraction of both (phycobiliproteins and chlorophyll) compounds. Therefore, the extraction may be affected by chemical or mechanical methods. Since the macroalga has a very high water content, the use liquid nitrogen to make the biomass firmer to achieve a better grinding, and hence, a better extraction seems to be advantageous. In this sense, the choice end up on the amount/type of contaminants (namely chlorophylls) extracted.

3.5 Evaluation of all optimized conditions

After the optimization of several parameters of the proposed alternative extraction, it was made a comparison between both the optimized conventional and alternative extraction (**Figure 31**). The conditions adopted are the time of extraction and solid-liquid ratio at 20 minutes and 0.7 (mass of fresh algae/volume of solvent), respectively (previously optimized). In this comparison with the conventional extraction, the type of solvent, its concentration and pH were optimized for the alternative extraction and evaluated. The use of [Ch]Cl is justified by its very good capability of extraction of phycobiliproteins, namely R-phycoerythrin, and non-extraction of other contaminants such as chlorophyll, as reported in **Section 3.2.2**. Moreover, its low price and low ecotoxicity namely for marine bacteria, such as *Vibrio fischeri* [87], make this IL even more interesting in the application for R-phycoerythrin extraction.

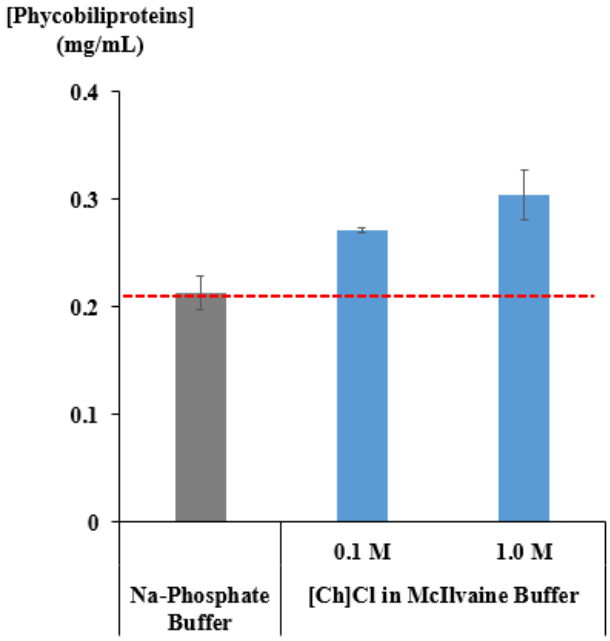

Figure 31. Comparison of the concentration of [Ch]Cl in the McIlvaine buffer in the phycobiliproteins extraction.

The experimental data in **Figure 31** shows that the extraction of phycobiliproteins increase with the increase of the concentration of [Ch]Cl, at least until 1 M. Moreover, the alternative extraction proposed increase the extraction in 30% when compared with the previously conventional methodology of extraction optimized, proving the success of the methodology.

3.6 R-phycoerythrin and contaminant proteins of the extracts

In order to truly identify the R-phycoerythrin and the nature and presence of potential contaminants, a SDS-PAGE assay was performed for the extracts obtained with the sodium phosphate buffer and with 0.1 M and 1.0 M of [Ch]Cl in McIlvaine buffer, as indicated in **Figure 32**.

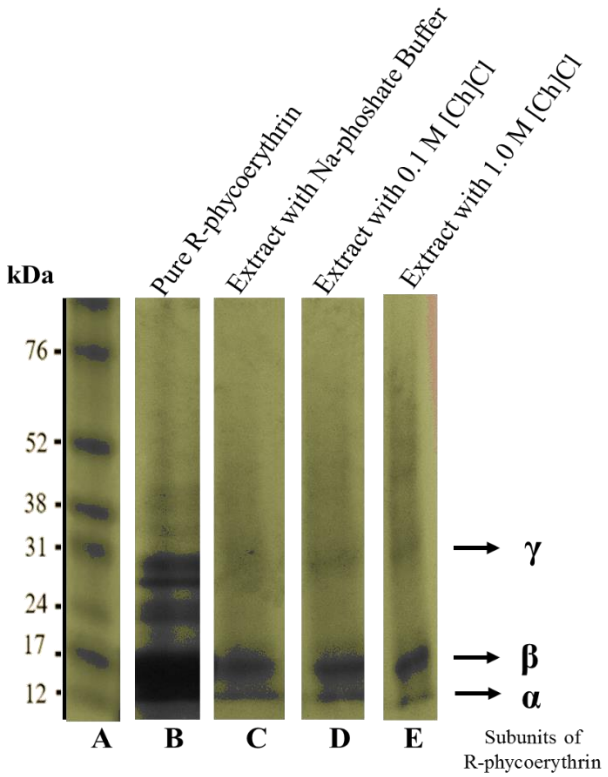

Figure 32. SDS-PAGE of phycobiliproteins extracted from *G. vermiculophylla*: **(A)** Protein marker; **(B)** Pure R-phycoerythrin; **(C)** extract obtained with sodium phosphate buffer; **(D)** extract obtained with 0.1 M of [Ch]Cl in McIlvaine buffer; and **(E)** extract obtained with 1.0 M of [Ch]Cl in McIlvaine buffer.

The crude extracts (**Figure 32**C, D, and E) were analysed and compared considering the same molecular-weight size marker **(Figure 32**A) and a solution of pure R-phycoerythrin (**Figure 32**B). Briefly, it is possible to identify the presence of R-phycoerythrin in all extracts by the presence of its subunits α, β, and γ, as already suggested before by the colour of the extracts and their absorption spectra.

4. CONCLUSIONS

In this work a new and alternative method for extraction of phycobiliproteins (namely R-phycoerythrin) was designed. Firstly, a conventional methodology using the sodium phosphate buffer as main solvent was optimized. The time of extraction and solid-liquid ratio, considering the fresh biomass, were fixed in 20 minutes and 0.7 (mass fresh alga/volume of solvent), respectively. Then, the screening of different ILs, salts and ILs + salts mixtures was done in order to optimize and identify the most promising solvent. The main results suggest that it is possible to extract different classes of compounds from *Gracilaria vermiculophylla*, namely phycobiliproteins and chlorophyll, using different ILs. This is mainly influenced by the length of the IL' cation: smaller alkyl chains lead to the phycobiliproteins extraction while longer alkyl chains allow the chlorophyll extraction. Focusing in the extraction of phycobiliproteins, it was found that regarding the ILs tested, [Ch]Cl is the best IL for the extraction of phycobiliproteins (and R-phycoerythrin), not due to its capacity in the cell disruption, but because of its capacity to increase the solubility of the proteins in water.

For the alternative extraction method, a surface response methodology was performed using a 2^3 factorial design aiming at the simultaneous study of the effect of the solid-liquid ratio, pH, and the salt concentration of the buffer evaluated. The McIlvaine buffer instead of sodium phosphate buffer since this allows the study in a wider range of pH values. Moreover, the use of the McIlvaine buffer was proved to be better than the sodium phosphate buffer in the phycobiliproteins extraction. Results of surface response methodology showed that, as seen before, the phycobiliprotein extraction increases with the increase of the solid-liquid ratio, however for very high solid-liquid ratios the extraction is experimentally hindered. Meanwhile, the extraction is not influenced by the salt concentration of the McIlvaine buffer but, at the same time, the pH showed to be a significant parameter being optimized and fixed at 5.9.

The alternative extraction using [Ch]Cl with the McIlvaine buffer and in aqueous solution (at pH = 5.9), showed to be 30% more efficient than the optimized conventional extraction.

5. FUTURE WORK

Considering the results achieved in the solid-liquid extraction task, it will be necessary to perform new studies regarding the fractionation of the phycobiliproteins and thus, the purification of each phycobiliprotein.

Given the preliminaries results for some ILs, in particular, the most hydrophobic ones which promote the extraction of chlorophyll, the study of the sequential extraction of R-phycoerythrin and chlorophyll would be of great interest to enhance the value and potential of this biomass.

6. LIST OF PUBLICATIONS - Scientific Contribution

Panel communications in International Conferences:

M. Martins, F.A. Vieira, M.H. Abreu, J.A.P. Coutinho, S.P.M. Ventura, Extraction of the antitumoral protein R-phycoerythrin from red macroalgae using ionic liquid-based extractive technologies, BPP 2015 - Biopartitioning & Purification Conference 2015, 7-10 June, 2015, Vienna, Austria.

M. Martins, F.A. Vieira, M.H. Abreu, J.A.P. Coutinho, S.P.M. Ventura, Using ionic liquids in the purification of the antitumoral protein R-phycoerythrin from *Gracilaria vermiculophylla*, IMIL 2015 - Iberoamerican Meeting on Ionic Liquids, 2-3 July, 2015, Madrid, Spain.

M. Martins, F.A. Vieira, M.H. Abreu, J.A.P. Coutinho, S.P.M. Ventura, Extraction of R-Phycoerythrin from Red Macroalgae using Ionic Liquids as Solvents – a bridge between sunlight and energy, 2nd EuCheMS Congress on Green and Sustainable Chemistry, 4-7 October, 2015, Lisbon, Portugal.

Oral communications in International Conferences:

J.H.P.M. Santos, M. Martins, J.A.P. Coutinho, S.P.M. Ventura, Development of a fractionation extractive platform of phenolic compounds from lignocellulosic biomass, 2nd EuCheMS Congress on Green and Sustainable Chemistry, 4-7 October, 2015, Lisbon, Portugal.

Papers:

M. Martins, F.A. Vieira, M.C. Neves, M.H. Abreu, J.A.P. Coutinho, S.P.M. Ventura, Extraction of phycobiliproteins from the red macroalgae *Gracilaria vermiculophylla* using aqueous solutions of ionic liquids, *in preparation*.

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8. APPENDIX

APPENDIX A: Calibration Curves

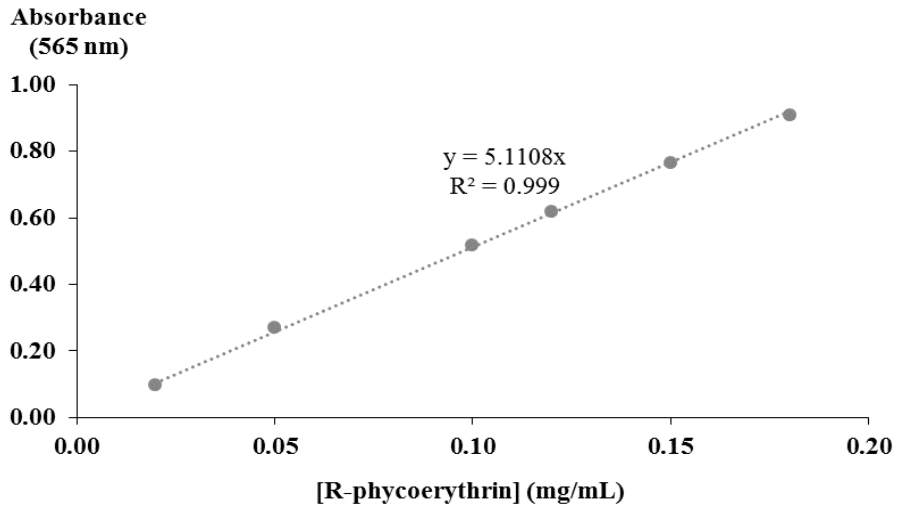

Figure A1. Calibration curve for R-phycoerythrin quantification in aqueous solution at 565 nm made with purified R-phycoerythrin solutions using a UV-Vis microplate reader (Synergy HT microplate reader – BioTek).

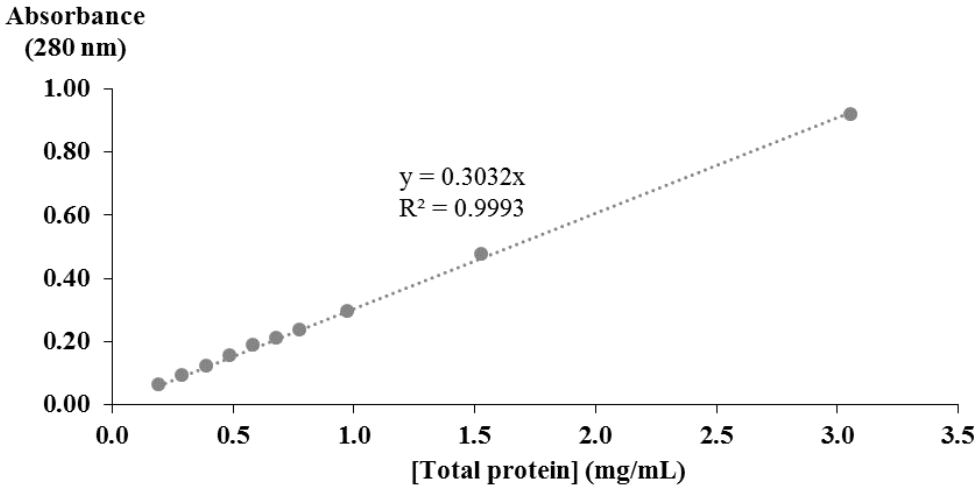

Figure A2. Calibration curve for total protein quantification in aqueous solution at 280 nm made with Bovine serum Albumin using a UV-Vis microplate reader (Synergy HT microplate reader – BioTek).

APPENDIX B: SRM Extra Data

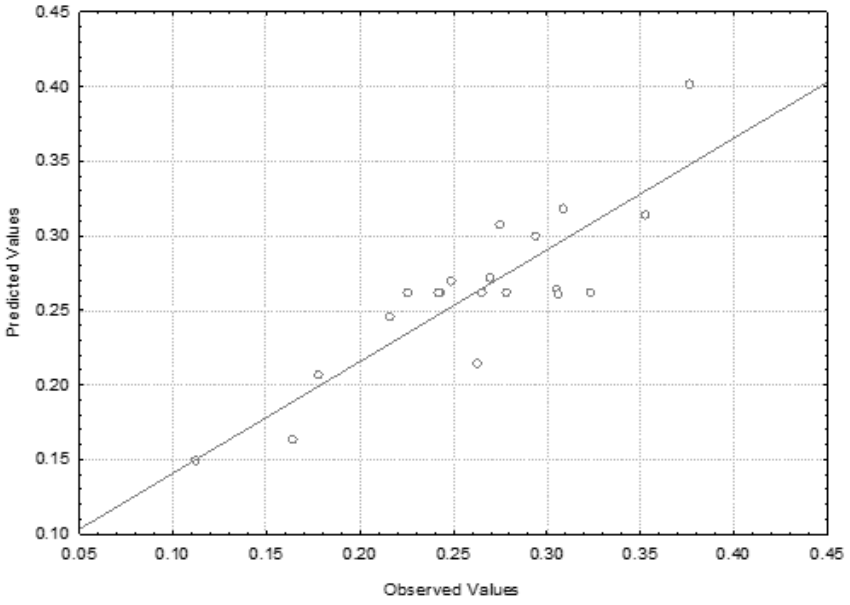

Figure B1. Predicted versus observed values for the factorial design of 2^3.

Table B1. Data attributed to the independent variables ([salt], pH and SLR) to define the 2^3 factorial planning for the system under study and respective results of concentration of phycobiliproteins extracted experimentally, the theoretical results found for the mathematical model developed and the respective relative deviation.

Run	[Salt] (M)	pH	SLR	[Phycobiliproteins] (mg/mL) Experimental values	[Phycobiliproteins] (mg/mL) Theoretic values	Residues
1	0.3	5.80	0.60	0.294	0.300	-0.005
2	0.7	5.80	0.60	0.306	0.264	0.042
3	0.3	8.20	0.60	0.268	0.214	0.049
4	0.7	8.20	0.60	0.164	0.163	0.001
5	0.3	5.80	0.80	0.353	0.314	0.039
6	0.7	5.80	0.80	0.309	0.318	-0.009
7	0.3	8.20	0.80	0.270	0.272	-0.001
8	0.7	8.20	0.80	0.306	0.261	0.046
9	0.2	7.00	0.70	0.217	0.246	-0.029
10	0.8	7.00	0.70	0.178	0.206	-0.028

11	0.5	5.00	0.70	0.249	0.269	-0.020
12	0.5	9.02	0.70	0.112	0.149	-0.037
13	0.5	7.00	0.53	0.275	0.307	-0.032
14	0.5	7.00	0.87	0.376	0.401	-0.025
15	0.5	7.00	0.70	0.243	0.261	-0.018
16	0.5	7.00	0.70	0.242	0.261	-0.019
17	0.5	7.00	0.70	0.324	0.261	0.062
18	0.5	7.00	0.70	0.226	0.261	-0.036
19	0.5	7.00	0.70	0.265	0.261	0.004
20	0.5	7.00	0.70	0.278	0.261	0.017

Table B2. Regression coefficient of the predicted second-order polynomial model for the phycobiliproteins extraction obtained from the RSM design using McIlvaine Buffer as solvent.

	Regression Coeffient	**Standard Deviation**	**t-student (10)**	***p*-value**
Interception	1.769	1.024	1.728	0.115
[Salt]	0.016	0.762	0.021	0.984
$[Salt]^2$	-0.313	0.288	-1.089	0.302
pH	0.095	0.148	0.641	0.536
pH^2	-0.013	0.008	-1.613	0.138
SLR	-5.203	1.890	-2.753	0.020
SLR^2	3.282	1.151	2.851	0.017
[Salt] x pH	-0.015	0.064	-0.240	0.815
[Salt] x SLR	0.496	0.772	0.643	0.535
pH x SLR	0.091	0.129	0.711	0.493

Table B3. ANOVA data for the extraction of phycobiliproteins obtained from the factorial design of 2^3 planning.

	Sum of Squares	**Degrees of Freedom**	**Mean of Squares**	**F_{calc}**	***p*-value**
Regression	0.055	9	0.006	3.179	0.038
Error	0.019	10	0.002		
Total	0.076	19	**R^2** = 0.7494	**$F_{9,10;0.05}$** = 3.02	